Fucose-containing sulfated polysaccharides from brown seaweed: Extraction technolgy and bioactivity assessment

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DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

Technical University of Denmark

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Preface

Enormous amounts of seaweed resource still remain unexploited. Examining new applications of this unexploited seaweed by developing state-of-the-art solutions; and innovation of seaweed products are the primary interest and motivation of this PhD study.

The PhD work was initiated at the Department of System Biology, Technical University of Denmark (DTU) and was completed at Center for Bioprocess Engineering (BioEng), Department of Chemical and Biochemical Engineering System Biology, Technical University of Denmark (DTU), Lyngby Denmark. The main research and experimental activities were performed in BioEng laboratory facilities located at Bldg. 227 DTU Søltoft Plads, Kgs. Lyngby. In fulfillment of the PhD programme various courses, seminars, conference participations and research activities were taken both locally and internationally. External research activity was also accomplished at School of Allied Health Sciences, Kitasato University, Sagamihara, Kanagawa, Japan.

In this PhD thesis, published works are cited and the chapters are based on published research and scientific papers completed by the author during the PhD study:

Chapter 1 is an introduction to the state-of-the-art FCSP methods. It also outlines the problems, hypotheses, and core objectives of this PhD study. This chapter provides an overall outline of different scientific investigations and research activities in the form of project phases.

Chapter 2 is based on Paper 1, which highlights FCSP structure-function relationships and extraction methods. It also includes an overview of seaweed potentials based on recent development and reports from published journals.

Chapter 3 is about optimized single-step extraction of FCSPs based on Paper 2. This chapter provides an in-depth investigation of the influence of different extraction parameters on the chemical nature and structural features of FCSPs.

Chapter 4 shows the FCSP bioactivity. The anti-proliferative and immune response activity of fucoidan extracted from *Sargassum* sp. using minimal processes based on Papers 3 and 4 is also presented.

Chapter 5 is based on Paper 5. Successful exploitation of seaweed for commercial applications is accomplished provided that growth parameters are optimized; hence, growth and nutrient assimilation monitoring of seaweed has also been the subject of this study using *U. lactuca* seaweed as a model.

Chapter 6 contains the final remarks about the present work. It also gives some future perspectives and prospective research areas.

This whole PhD study was undertaken with superior guidance and supervision of Prof. Anne S. Meyer, head of Center for Bioprocess Engineering and tireless encouragement of Prof. Jørn Dalgaard Mikkelsen, Center for Bioprocess Engineering as co-supervisor. Moreover, the diligent assistance and supervision of Dr. Hiroko Maruyama and Dr. Hidezaku Tamauchi during my research work in Kitasato University, Kanagawa Japan was so beneficial for the advancement of this PhD study.

The PhD project was fully financed by the Technical University of Denmark (DTU) for a period from March 2008 to October 2011.

This thesis is submitted for the fulfillment of the PhD degree requirements at Technical University of Denmark.

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Technical University of Denmark (DTU)
February 2012

Abstract

Marine seaweed that is washed up on the coastline is a nuisance as its degradation produces a foul a smell and generates waste problems. Exploitation of coastline-polluting seaweeds such as *Sargassum* sp., *Ulva* sp., and other beach-cast seaweed species for various commercial applications will generate new valuable products that may help lessen coastal pollution by seaweeds and create new seaweed-based resources. Thus, utilization of these natural resources is of great importance. The objectives of this PhD study were to develop a technology to extract bioactive compounds from nuisance brown seaweeds, and investigate their bioactivity. To this effect, designed optimized extraction of fucose-containing sulfated polysaccharides (FCSPs) and/or crude fucoidan from brown seaweed were performed, and the bioactivity of the isolated FCSPs was investigated. Moreover, to assess the potential of seaweed to assimilate nitrogen-based nutrients, a technology for accurate monitoring of differential seaweed growth responses to nutrient assimilation was also developed.

Fucoidan is a term used to describe a class of sulfated polysaccharides extracted from brown seaweed, which contains substantial amounts of fucose; varying amounts of galactose, xylose, and glucuronic acid; and differing glycosidic linkages, and are variously substituted with sulfate and acetyl groups and side branches containing fucose or other glycosyl units. These FCSPs principally consist of a backbone of (1→3)- and/or (1→4)-linked α-L-fucopyranose residues that may be substituted with sulfate (SO_3^-) on C-2, C-3, or C-4 and acetyl groups at C-4 on the main chain or may have short fucoside side chains that are usually linked from the *O*-4 of one or several of the fucopyranose backbone residues. FCSPs are known to exhibit crucial biological activities including anti-tumor activity. Although differently extracted, purified, fucose-rich, modified fucoidans have been reported to exert bioactive properties such as anti-coagulant and enhance immune response activity, few studies have investigated the bioactivity of unfractionated FCSPs, notably FCSPs extracted using milder and fewer processing steps. Crude fucoidan from *Sargassum* sp. and *Fucus vesiculosus* were examined for their bioactivity against lung and skin cancer cell lines in both *in vitro* and *in vivo* studies. This study showed that unfractionated FCSPs hinder the *in vitro* proliferation of Lewis lung carcinoma and melanoma B16 cell lines by induction of apoptosis. Moreover, the anti-tumor activity of crude fucoidan seems to be associated with an enhanced immune response as depicted by an increase in natural killer cell activity in mice.

The classical extraction of FCSPs involving long, repetitive, multi-step acid and alkaline treatments is detrimental to its structural properties, yield, and compositional attributes. In this study, statistically designed, optimized extraction of a single-step extraction of FCSPs from *Sargassum* sp. was carried out. The effects of the different extraction parameters on the natural chemical composition of the isolated sulfated polysaccharides were also investigated. The data showed that classical multi-step extraction using ≥0.2 M HCl at elevated temperature and extended time had a detrimental effect on the FCSPs yield, as this treatment apparently disrupted the structural integrity of the polymer and evidently degraded carbohydrate chains of fucose residues during extraction. The results also revealed a maximal FCSPs yield of approximately 7% dry weight with *Sargassum* sp. using 0.03 M HCl at 90°C and 4-h extraction conditions.

Accurate monitoring of the differential growth response of seaweed to different nutrient assimilation is crucial to explore various applications of seaweed resources, such as biomass for bioenergy production and source of functional healthy components and bioactive compounds. A major prerequisite for the successful exploitation of cultivated seaweed like *Ulva lactuca* for commercial purposes is that the growth rate and yields should be optimized. In this study, the growth response of *U. lactuca* to ammonium and nitrate assimilation was investigated using a photoscanning technique to monitor the growth kinetics in *U. lactuca*. Photoscanning images revealed differential increases in the surface area of *U. lactuca* discs over time in response to different nitrogen-based nutrient sources. The results also showed a favorable growth response to ammonium as a nitrogen source, and the presence of ammonium discriminated the nitrate uptake by *U. lactuca* upon exposure to ammonium nitrate. This study exhibits the applicability of a photoscanning approach for acquiring precise quantitative growth data for *U. lactuca*.

In conclusion, we demonstrated that nuisance seaweed can be a potential source of biomass and bioactive compound notably FCSPs. This study proved the hypotheses that different extraction conditions have crucial influenced to the chemical nature of FCSPs. The study also demonstrated that unfractionated FCSPs are able to exert bioactive actions such as anti-tumor and immune-modulating properties in both *in vitro* and *in vivo* studies. This study illustrates the importance of a precise monitoring technique of the growth of *U. lactuca* in order to successfully exploit it for commercial application.

Dansk Sammenfatning

Tang på stranden er en plage, først og fremmest på grund af de lugtgener, som kommer når tangen går i forrådnelse. En udnyttelse af dette tang, såsom *Sargassum* sp., *Ulva* sp., og andre lignende kyst-nære typer af tang, kan frembringe helt nye værdifulde produkter og måske samtidig mindske de uønskede lugtgener fra tang som skyller op på stranden. Udnyttelse af tang som resource er udgangspunktet for dette PhD studium. PhD studiets formål har været dels at udvikle en ekstraktionsmetode til at isolere bioaktive produkter fra brunt tang, dels at undersøge disse produkters bioaktivitet. For at opfylde dette formål blev der i PhD arbejdet udviklet en statistisk designet, optimeret ekstraktionsmetode til ekstraktion af såkaldte fukose-indeholdende sulfaterede polysakkarider (*eng*. fucose-containing sulfated polysaccharides), forkortet FCSPs, henholdsvis grov fucoidan fra brun tang, primært *Sargassum* sp., og bioaktiviteten af ekstraktionsproduktet blev undersøgt. For ydermere at vurdere tangs evne til at assimilere, og dermed vokse på nitrogen-holdige salte, blev der udviklet en teknologi til at monitorere differential vækst af *Ulva lactuca*, på forskellige næringsstoffer i vandet.

Fucoidan er en betegnelse, som dækker over en gruppe af sulfaterede, fukose-holdige, polysakkarider fra tang. Udover fukose indeholder fucoidan forkellige mængder galaktose, xylose, glukuronsyre, som er forbundet via forskellige typer glykosidbindinger, og som derudover er substitueret i forskellig grad med sulfat og acetyl-grupper og som kan have sidekæder indeholdende fukose eller andre glykosyl-substituenter. Disse FCSPs består principielt af en rygrad, eller en hovedkæde, af (1→3)- og/eller (1→4)-linkede α-L-fucopyranose enheder, som kan være substitueret med sulfate (SO_3^-) på C-2, C-3, eller C-4 foruden acetyl grupper på C-4 på fucose-enhenderne i hovedkæden, og/eller som har korte fukose-kæder, der normalt er bundet via *O*-4 fra en eller flere fukose-enheder i hovedkæden. Det er kendt, at FCSPs isoleret fra tang har forskellige gavnlige, bioaktive effekter, herunder anti-tumor aktivitet. Selvom det har været rapporteret, at oprensede, fucose-rige, modificerede fucoidan-prøver har bioaktive effekter, såsom anti-koagulerende, og immun-respons forøgende egenskaber, er bioaktiviteten af mere grove, ufraktionerede FCSPs – ekstraheret med mildere og færre ekstraktionstrin - ikke undersøgt.

Groft oprensede fucoidan-prøver fra *Sargassum* sp. og *Fucus vesiculosus* blev i dette PhD arbejde undersøgt for deres bioaktivitet mod lunge- og hudcancer cellelinjer vækst både *in vitro* og *in vivo*.

Arbejdet viste, at ufraktionerede FCSPs ekstrakter hindrer proliferation in vitro af Lewis lung carcinoma og melanoma B16 celle linjer via induktion af apoptosis. Desuden blev det vist, at denne anti-tumor aktivitet af grov fucoidan, tilsyneladende er associeret med et øget immunrespons, målt som et forøget niveau af naturlige "killer" cellers aktivitet i mus.

Klassisk ekstraktion af FCSPs fra tang involverer adskillige langsommelige, behandlinger med syre og base, hvilket er ødelæggende for deres specifikke struktur og sammensætning. I dete PhD arbejde blev der udviklet en statistisk designet, optimeret enkelt-trins ekstraktionsmetode til at udtrække FCSPs fra *Sargassum* sp.. Effekten af de forskellige ekstraktionsparametre på sammensætningen af de ekstraherede polysaccharider belv også vurderet. Resultaterne viste, at klassisk, multi-trins ekstraktion ved brug af ≥0.2 M HCl ved høj temperature havde en ødelæggende effect på udbyttet af FCSPs og viste desuden at en sådan behandling tilsyneladende ødelagde polysaccharidstrukturen og at fukose-kæderne blev nedbrudt under behandlingen. Resultaterne viste også, at et maximal FCSPS udbytte, på ca. 7% af tørvægten for *Sargassum* sp. kunne opnås ved et-trins ekstraktion med 0.03 M HCl ved 90°C i 4 timer.

Nøjagtig monitorering af vækstrespons af tang på forskellige næringsstoffer er afgørende for at undersøge forskellige anvendelser af tang, primært biomassevækst til bioenergi-produktion og til udnyttelse af tang som kilde til produktion af f.eks. funktionelle fødevarekomponenter eller bioaktive stoffer. En vigtig forudsætning for kommerciel udnyttelse af kultiveret tang såsom *Ulva lactuca* er, at vækstraten og udbyttet optimeres. I dette studie blev vækstrespons af *U. lactuca* I forhold til ammonium og nitrat-assimilation undersøgt ved hjælp af en foto-scannings teknik. Foto-scannede billeder afslørede forskellige vækst-inkrementer udfra måling af det fotograferede areal af udstukne skiver af *U. lactuca* over tid som respons på forskellige nitrogen-baserede næringskilder. Resultaterne viste også en favorabel vækstøgning på ammonium som nitrogenkilde, og desuden at tilstedeværelsen af ammonium diskriminerede *U. lactucas* nitrat-optag i forhold til optaget af ammonium nitrat. Studiet viste anvendeligheden af phot-scanning til præcis kvantitativ vækst-monitorering af *U. lactuca*.
Den samelde konklusien er således at vi demonstrerede, at tang kan være en potential kilde til biomasse og bioaktive komponenter, især FCSPs. PhD studiet viste desuden at hypotesen, at forskellgie ekstraktionsbetingelser har afgørende indflydelse på den kemiske natur af FCSPs er sand. Studiet demonstrerede også, at ufraktionerede FCSPs har bioaktive egenskaber såsom anti-tumor og immun-modulerende effecter, vist i både *in vitro* og *in vivo*. Studiet ilustrerer desuden vigtigheden af en præcis monitoreringsmetode til måling af *U. lactuca* vækst.

List of publications

The PhD thesis is based on the work presented in the following papers:

I. Important determinants for fucoidan bioactivity: A critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweed
Ale MT, Mikkelsen JD and Meyer AS
Marine Drugs (2011) 9, 2106 - 30, DOI:10.3390/md9102106, **Published online**

II. Designed optimization of a single-step extraction of fucose-containing sulfated polysaccharides from *Sargassum* sp.
Ale MT, Mikkelsen JD and Meyer AS
Journal of Applied Phycology (2011)
DOI: 10.1007/s1081-011-9690-3, **Published online**

III. Fucoidan from *Sargassum* sp. and *Fucus vesiculosus* reduces cell viability of lung carcinoma and melanoma cells *in vitro* and activates natural killer cells in mice *in vivo*
Ale MT, Maruyama H, Tamauchi H, Mikkelsen JD and Meyer AS
International Journal of Biological Macromolecules 49 (2011) 331 – 336
DOI: 10.1016/j.ibiomac.2011.05.009, **Published online**

IV. Fucose-containing sulfated polysaccharides from brown seaweed inhibit proliferation of melanoma cells and induce apoptosis by activation of caspase-3 *in vitro*
Ale MT, Maruyama H, Tamauchi H, Mikkelsen JD and Meyer AS
Marine Drugs (2011) 9, 2605 - 21 DOI:10.3390/md9122605, **Published online**

V. Differential growth response of *Ulva lactuca* to ammonium and nitrate assimilation
Ale MT, Mikkelsen JD and Meyer AS
Journal of Applied Phycology (2011) 23: 345–351, **Published**

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1 Introduction

In the vast coastal areas worldwide, seaweed has played a major role in maintaining the ecological balance of the environment and marine bio-ecosystem. Seaweeds, including various brown seaweeds such as *Undaria* and *Laminaria* spp., are part of the food culture in many Asian countries, notably Japan, the Philippines, and Korea, and seaweed extracts have also been used as remedies in traditional medicine. In recent years, harvesting and monoculture farming of certain seaweed species have become an important livelihood for fishermen in Southeast Asia owing to the progressively increasing demand for raw seaweed worldwide. Many seaweed species, notably beach-cast seaweeds, still need to be examined for their characteristics and properties, which will determine their commercial applications, before they can be recognized as important commodities.

Seaweed that washes up on the coastline often generates waste problems for populations residing seaside owing to microbial accumulation and unpleasant odors. Utilization of beach-cast seaweeds such as *Ulva* sp., *Sargassum* sp., and other nuisance seaweed species for advantageous applications may alleviate these problems and create valuable seaweed-based products. It is widely known that seaweed may contain unique components that have potential commercial applications; however, few seaweed species are commercially utilized and others remain unexploited. Some seaweed species could be potential sources of functional dietary fiber and polysaccharides with bioactive properties (Lahaye 1991; Takahashi 1983). Recent developments in seaweed utilization include applications involving naturally derived seaweed extracts and bioactive compounds such as fucose-containing sulfated polysaccharides (FCSPs) and/or fucoidan in some cosmetic products and food supplements.

Fucoidan is a term used to described a class of sulfated polysaccharide extracted from the seaweed class Phaeophyceae, which consist almost entirely of fucose and ester sulfate (Percival and McDowell 1967). This FCSP principally consists of a backbone of (1→3)- and/or (1→4)-linked α-L-fucopyranose residues that may be organized in stretches of (1→3)-α-fucan or with alternating α(1→3)- and α(1→4)-bonded L-fucopyranose residues. The L-fucopyranose residues may be substituted with sulfate (SO_3^-) on C-2 or C-4 (rarely on C-3) single L-fucosyl residues and/or short fucoside (fuco-oligosaccharide) side chains. If present, the fucoside side chains are usually *O*-4, linked to the α-L-fucopyranose backbone residues. Apart from variations in the sulfate content and

substitutions, the monosaccharide composition of FCSPs varies among different species of brown seaweeds. Hence, in addition to fucose, different types of FCSPs may also contain galactose, mannose, xylose, glucose, and/or glucuronic acid, usually in minor amounts (Percival and McDowell, 1967; Bilan and Usov, 2008). It appears that FCSPs cover a broader range of complex polysaccharides than those having only fucan backbones. Fucoidan—or more correctly, FCSPs extracted from brown seaweeds like *Sargassum* sp. and *Fucus vesiculosus*—were documented to have a wide range of biological activities including anticoagulant (Nardella et al., 1996); anti-inflammatory (Blondin et al., 1994); antiviral (Adhikari et al., 2006; Trinchero et al., 2009); and, notably, anti-tumoral effects (Zhuang et al., 1995; Ale et al., 2011).

Typical extraction of FCSPs from brown seaweed involves a harsh processing condition and several purification steps. Purification of FCSPs by column chromatography was effective for isolating polysaccharide fractions as they had higher fucose contents than those FCSPs obtained using minimal processing steps (Li et al., 2006; Ale et al., 2011). The conditions for obtaining FCSPs from brown seaweed generally consist of multiple, long, repetitive steps using acid (e.g., HCl) and other solvents at elevated temperatures (Chizhov et al., 1999; Bilan et al., 2002). The influence of the extraction methods on the chemical nature of sulfated polysaccharide has already been demonstrated by Black et al. (1952). On the other hand, the FCSP yield of *F. evanescens* extracted 4 times using 2% $CaCl_2$ solution at 85°C for 5 h was 12.9% dry weight (DW) (Bilan et al., 2002), while extraction at 25°C using 0.4% HCl for 5 h yielded 12.0% DW (Zvyagintseva et al., 1999). Despite the existence of early seminal studies about FCSP or fucoidan extraction, there is only limited evidence about the influences and apparently complex interactions of extraction parameters, such as acid solvents, temperature, and time, on FCSP yield and composition. FCSP extraction procedures with fewer steps are milder on the brown seaweeds than are other though they may yield a heterogeneous sulfated polysaccharide product. Nevertheless, fewer steps extraction approach minimizes the structural alteration of algal-sulfated polysaccharides and, thereby, maintains the natural bioactive characteristics of FCSPs.

Although differently extracted and purified FCSPs have been reported to exert bioactivity (Holtkamp et al., 2009), unfractionated FCSPs has also been found to reduce cell proliferation of lung carcinoma and melanoma cells, exert immunopotentiating effects in tumor-bearing animals, and to activate natural killer (NK) cells in mice, leading to anti-tumor activity efficacy (Takahashi, 1983; Ale et al., 2011; Foley et al., 2011). Kim et al. (2010) applied a crude polysaccharide composed predominantly of sulfated fucose from *F. vesiculosus* to human colon cancer cells *in*

vitro and concluded that this polysaccharide from brown seaweed induces apoptosis. Moreover, commercially available crude fucoidan (Sigma Inc.) was tested on human lymphoma HS-Sultan cell lines and was found to inhibit proliferation and induce apoptosis by activating caspase-3 (Aisa et al., 2005). It was reported recently that FCSPs from *Sargassum* sp. and crude fucoidan (Sigma Inc.) from *F. vesiculosus* induced apoptosis in melanoma cells (Ale et al., 2011).

Besides the bioactive compounds, seaweeds also possess other valuable components, such as soluble dietary fibers and carbohydrates for hydrocolloid applications. The green seaweed (*Ulva* species) are particularly rich in rare cell wall polysaccharides and have been proposed as being important sources of dietary fiber, mainly soluble fiber (Lahaye 1991; Lahaye and Axelos 1993). Furthermore, *U. lactuca* is also a good source of vitamins A, B2, B12, and C and is rich in γ-tocopherol (Abd El-Baky et al., 2008; Ortiz et al., 2006). It has been shown that seaweed, notably *U. lactuca,* was suitable for propagation under controlled conditions (Vermaat and Sand-Jensen, 1987; Lee, 2000; Sato et al., 2006). For this reason, *U. lactuca* cultivation in tanks for either crude biomass production for bioenergy or for the production of biologically active compounds is currently receiving increased attention (Hiraoka and Oka, 2008). However, a major prerequisite for the successful exploitation of cultivated *U. lactuca* for commercial applications is optimization of growth rates and yields. This in turn requires both an understanding of the influence of different nutrients on the growth response and a precise methodology to measure the growth. Nuisance green seaweeds like *U. lactuca* showed bioremediation ability in nitrogen- and phosphate-rich waste water (Copertino et al., 2008). Nevertheless, limited information is known about the growth response and nutrient uptake assimilation of *U. lactuca* when expose to combine concentrations of ammonium and nitrate.

This PhD thesis delivers the most recent study involving FCSPs extraction technology and evaluates FCSPs biological activity. A single-step extraction method for the removal of FCSPs from *Sargassum* sp. was developed in the course of this study. FCSPs bioactivity studies have been conducted in lung and skin cancer cell models *in vitro* and immune response activity *in vivo*. In addition to extraction and bioactivity studies, evaluation of the growth response of *U. lactuca* to nutrients such as NH_4 and NO_3 was also performed.

1.1 Problems statement

Purified fractions from FCSPs are commonly used for structural and bioactivity analysis. These samples contain high fucose-sulfate levels and are free from other contaminant saccharide

residues. Hence, many seminal studies have shown that purified fucoidan has high bioactivity as a result of multi-step extraction and further purification and fractionation.

- Can an unpurified FCSPs product extracted using minimal steps exert bioactivity?
- What is the effect of unpurified FCSPs against certain cancer cell lines and what is its influence on immune response activity?

The present extraction technology using aqueous-alkali solution or dilute acid at ambient or slightly elevated temperature has always been the most convenient method to produced FCSPs.

- Would different extraction parameters, i.e., acid, temperature, and time, influence the structural features and chemical nature of FCSPs?
- What are the effects of the interactions of different extraction treatments?

Exploitation of seaweed resources has recently received special attention for its potential for both the production of bioactive compounds and as a biomass source for bioenergy production. To successfully exploit seaweed for commercial applications, the growth rate and yields must be optimized. Hence, a precise monitoring technology to evaluate the seaweed growth response is required.

- What are the different monitoring techniques that are used to evaluate the *U. lactuca* growth response to nutrient assimilation? How does it affect the measurements' precision?
- How is the growth of *U. lactuca* influenced by the assimilation of different nutrients (NH_4 and NO_3)?

1.2 Hypotheses

Nuisance marine seaweed that has washed up on the coastlines is a potential starting material for producing bioactive compounds like fucoidan or FCSPs. The precise assessment of seaweed growth is crucial in our understanding of seaweed nutrient assimilation mechanisms. In this thesis, some specific hypotheses were outlined:

- Minimal extraction step of FCSPs will preserve the polysaccharides' structural integrity and, thus, increase the yield and improve its biological properties

- FCSP products from a single-step extraction process (i.e., crude fucoidan) from brown seaweed can exert bioactivity against certain types of cancer cell lines (e.g., by inducing apoptosis) and can promote immune responses
- Classical extraction of FCSPs involving long, repetitive, multi-step acid and alkaline treatments can be detrimental to its structural makeup, yield, and compositional attributes and, thus, may influence its bioactive properties
- To exploit marine seaweed for commercial applications, growth monitoring is crucial to the accurate evaluation of differential growth responses and nutrient (NH_4 or $NaNO_3$) assimilations

1.3 Core objectives and project phases

Seaweed resources generally cover wide potential applications for different industries including food and nutrition, pharmaceuticals, cosmetics, and bioenergy. Therefore, their study requires extensive in-depth research involving an interdisciplinary approach and a considerable amount of time to accomplish certain achievable objectives. This PhD study narrows the subjects into more focused areas with very realistic aims or specific objectives to produce novel technology while attaining basic scientific understanding.

To test the hypotheses, various specific objectives were applied in this study. The core objectives were primarily concentrated on the investigation of different FCSPs extraction methods and developing innovative FCSPs extraction technology; the potential of seaweeds as a source of bioactive compounds, notably FCSPs; and to assess the bioactivity and mechanism of FCPS products against certain types of cancer. Furthermore, we evaluate seaweeds' differential growth responses to nutrient assimilation. Along with the core objectives, the specific aims of the study were as follows:

- Development of a new process for producing bioactive compound like fucose-containing sulfated polysaccharides from brown seaweed by optimized designed extraction parameters using of state-of-the-art analytical methods and quantification analyses
- Investigation of the bioactivity of single-step extracted unpurified FCSPs products (i.e., crude fucoidan) in cancer cell lines using *in vitro* and *in vivo* experiments
- Evaluation of the seaweed growth response to nutrient assimilation (i.e., NH_4 and NO_3)

To ensure that the work was conducted in the right direction, workloads were organized and narrowed down to specific scopes in a number of project phases:

Phase 1: **Survey of seaweed bioactive compound structure and bioactivity**

A literature research and gathering of review articles was conducted with the aim of gaining an overall perspective of the subject. Special focus was placed on the available technical and scientific information regarding bioactive compounds' structural makeup and composition, the influence of the extraction process, and factors affecting bioactivity.

Phase 2: **Optimized extraction and hydrolysis of seaweed polysaccharides (i.e., FCSPs)**

Fucoidan can be obtained by extraction from brown seaweeds like *Sargassum* sp. The typical extraction of FCSPs involves multi-step extraction using different temperatures and acid and alkaline concentrations in an extended period. In this study, it was hypothesized that harsh extraction treatment is detrimental to the integrity of the chemical nature of these polysaccharides; thus, preservation of its structure during extraction can only be done using a mild extraction technique. The main objective of this project was to design an optimized extraction of FCSPs using a minimal-step method compared to existing classical extraction methods with special attention placed on the influence on chemical composition.

Phase 3: **Assessment of FCSP bioactivity**

FSCPs are known to possess bioactive properties such as anti-tumor activity, apoptosis induction in cancer cells, and immune potentiation. The efficacy of FCSPs derived from brown seaweed remains a matter of debate; nevertheless, several reports attribute its efficacy to its structural makeup, substitutions, and content of sulfate. It was hypothesized that mild extracted FCSPs must possess high bioactive properties because their structural makeup remains intact. The objective of this study was to examine the different contributing factors influencing FCSP bioactivity on some cancer cell lines. The bioactivities of FCSPs extracted from nuisance seaweed were evaluated using cancer cell lines *in vitro* and *in vivo* experiments.

Phase 4: **Seaweed growth response to nutrient assimilation**

The proliferation of seaweed is mostly influenced by photosynthesis and available nutrients that can be utilized and assimilated. Seaweed growth is tantamount to increased biomass production. Seaweed can potentially be utilized for the production of bioactive compounds and healthy

components. Monitoring the seaweed growth and nutrient uptake is a crucial step for evaluating the seaweed growth rate, nutrient uptake rate, and biomass yield. Thus, developing a method to properly monitor seaweed growth will contribute to our understanding of the seaweed growth response to nutrient and assimilation patterns. The objective of this study is to examine the *U. lactuca* growth response to nutrients, i.e., NH_4 or $NaNO_3$, with the special aim of developing a growth monitoring technique to accurately evaluate the differential growth response and nutrient assimilation and to analyze different biomass accumulation-related parameters.

2 Seaweed potentials: a general overview

Marine seaweeds are classified according to their morphology and taxonomic characteristic into 3 groups: the green (Chlorophyta), red (Rhodophyta), and brown algae (Phaeophyta). The green algae are distributed worldwide (Lahaye & Robic, 2007), and the most common green seaweed species in the temperate zones is *Ulva lactuca* (Fig. 2.1). It is harvested naturally or cultivated in tanks for food consumption or biomass production for bioenergy (Lahaye & Robic, 2007). Natural habitats of red seaweed are found at intertidal and subtidal depths. In recent years, fishermen in the Southeast Asian countries engaged in the farming of the red seaweeds *Kappaphycus* and *Eucheuma* (Fig. 2.1) for the production of carrageenan. Carrageenan is a gel-forming, viscosifying polysaccharide that is commercially exploited for food products and cosmetic products (De Reiter and Rudolph, 1997). The brown seaweeds are usually found in tidal splash zones or rock pools, and certain species such as Sargassum are found floating on the shorelines (Fig. 2.1). Brown algae are typically used for the production of alginate, which is commercially used as an ingredient for different industrial, biotechnology, and food applications. FCSPs from brown seaweed, notably fucoidan, have been known to exert bioactive properties.

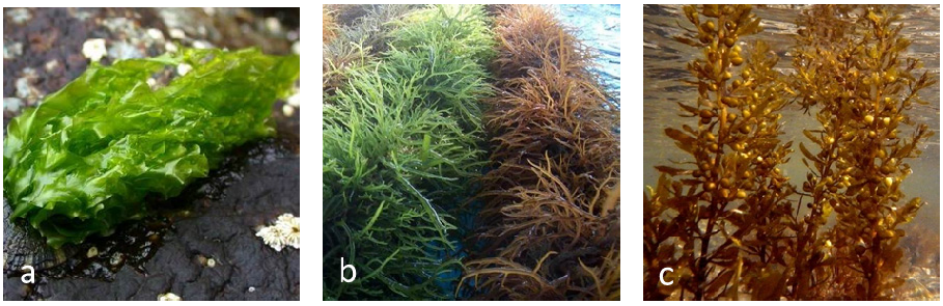

Fig. 2.1 Photographs of different seaweed species that are utilized for different commercial applications. a. *Ulva lactuca* as bulking agent for feeds. b. *Kappaphycus* sp. for carrageenan production. c. *Sargassum* sp. as a potential source of bioactive fucose-containing sulfated polysaccharides, particularly fucoidan compound. (Source: www.algaebase.org)

Several studies have indicated that FCSPs from brown seaweeds are highly potent anti-cancer agents, tumor cell growth inhibitors, and immune system strengtheners (Jiao et al., 2011; Pereira

et al., 1999). It has been suggested that the lower breast cancer and obesity rates in Japan may be related to regular intake of brown seaweed (Teas 1983). Brown seaweeds are very abundant and constitute unexploited resources worldwide, thus making them viable sources of polysaccharides, especially those with bioactive properties such as fucoidan or other FCSPs. Nevertheless, the red and green seaweeds are also good sources of functional food ingredients, including phycocolloids and dietary fibers.

2.1 Bioactive seaweed compounds: sulfated polysaccharides

Sulfated polysaccharides are a family of compounds containing ester sulfate groups in their sugar residues. These polysaccharides are commonly found in the marine algal groups Phaeophyta, Rhodophyta, and Chlorophyta. Fucans is the general term for sulfated polysaccharides that are present in the class Phaeophyta, which includes FCSPs. The sulfated polysaccharides in the class Rhodophyta are galactans consisting entirely of galactose or modified galactose units such as agar and carrageenans. The major polysaccharides in the class Chlorophyta are polydisperse heteropolysaccharides known as ulvans.

Ulva, the green seaweed species of the class Chlorophyta, are particularly rich in rare cell wall polysaccharides and have been proposed as being an important source of dietary fiber, mainly soluble fiber, which could be a potential prebiotic substrate (Lahaye 1991; Lahaye and Axelos 1993). Brown seaweed is a source of a unique compounds, notably FCSPs, which have shown high potency against certain cancers in some *in vitro* studies and exhibited immune response activity *in vivo* (Cumashi et al., 2007; Maruyama et al., 2003). Red seaweed, on the other hand, is known for its hydrocolloid characteristics; however, published reports have shown that sulfated polysaccharides from red seaweed also possessed some bioactive properties (Pereira et al., 2005; Talarico et al., 2007).

2.1.1 FCSPs structure

Fucoidan, an FCSP that is extracted from brown algae, may contain differing glycosidic linkages and is variously substituted with acetate and side branches containing fucose or other glycosyl units. Brown seaweed in the order of Fucales such as *F. evanescens* and *F. serratus* possess a large proportion of both $\alpha(1\rightarrow3)$- and $\alpha(1\rightarrow4)$-linked L-fucopyranose residues may be substituted with sulfate (SO_3^-) on C-2 and C-4 (Fig. 2.2; Bilan et al., 2002, 2006; Cumashi et al., 2007). The alga *Ascophyllum nodosum* (Fucales) has a predominant repeating structure $\alpha(1\rightarrow3)$-L-fucopyranose residues with sulfate at C-2 position-linked $\alpha(1\rightarrow4)$-L- fucopyranose residues with disulfate at C-2

position-linked α(1→3), the same structural elements that are also in FCSPs from *F. vesiculosus* (Fig. 2.2; Chevolot et al., 1999).

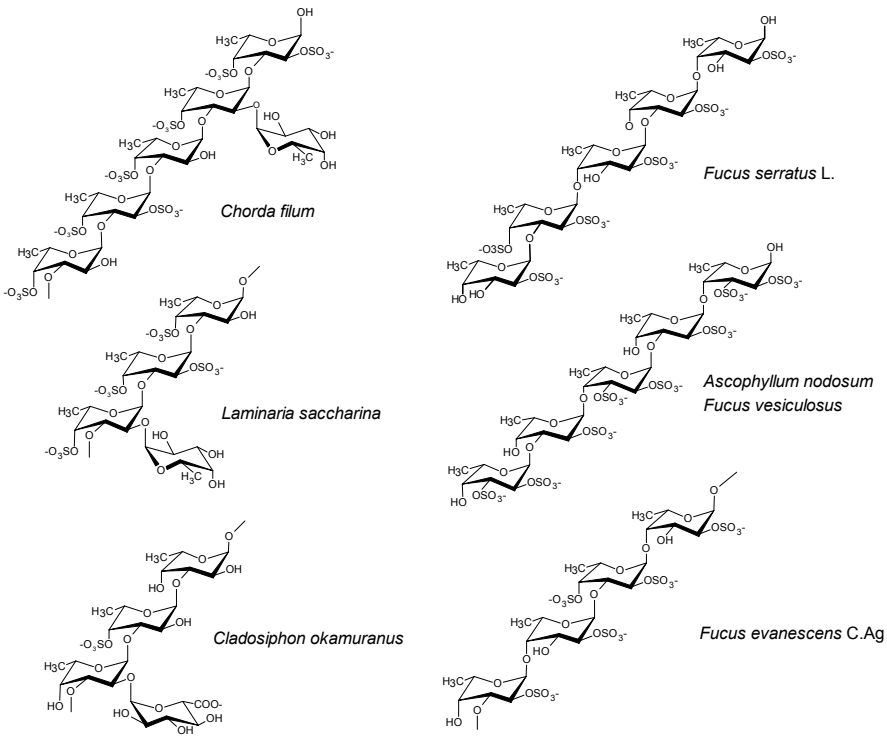

Fig. 2.2 Structural motifs of fucose-containing sulfated polysaccharides obtained from different brown seaweed species of the order Laminariales, Chordariales, and Fucales (Chizhov et al., 1999; Usov et al., 1998; Nagaoka et al., 1999; Chevolot et al., 2001; Bilan et al., 2002; 2006).

Several structures of fucoidans of the order of Laminariales were reported to contained monosulfate components that mainly consist of α(1→3)-linked L-fucopyranose residues with sulfates at the C-2 position (Berteau and Mulloy, 2003; Anastyuk et al., 2009). Fucoidan isolated from *Chorda filum* (Laminariales) has a structure of poly-α(1→3)-linked L-fucopyranose backbone and residues are sulfated mainly at C-4 and sometimes C-2 position, whereas some α(1→3)-linked fucose residues (Fig. 2.2) to be 2-*O*-acetylated (Chizhov et al., 1999). Similar structure of *L.*

saccharina (Laminariales) composed of α(1→3)-linked L-fucopyranose with sulfate at C-4 (Fig. 2.2) was previously reported by Usov et al., (1998). A structural study of fucoidan from *Cladosiphon okamuranus* (Chordariales) showed a linear backbone of α(1→3)-linked L-fucopyranose with a portion of fucose residues that was *O*-acetylated and sulfate substitution at the C-4 position (Fig. 2.2). It also contained α-glucuronic acid at 2 positions of fucose that were not substituted by a sulfate group (Nagaoka et al., 1999).

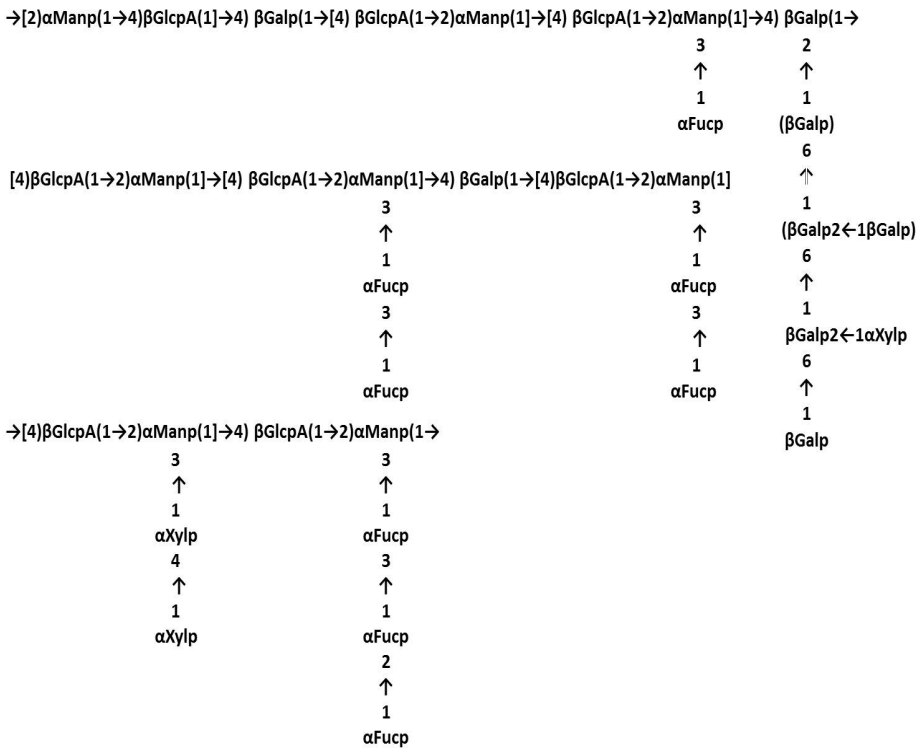

Fig. 2.3 Probable structure of fucoidan fraction from *Hizikia fusiforme* (Fucales) suggested by Li et al. (2006); structural composition was typical for *Sargassum* species (Duarte et al., 2001).

Complex fucoidan structures such as sulfated galactofucans and heterofucans brown algae have been reported (Bilan and Usov, 2008). A complex mixture of polysaccharides extracted from *Sargassum stenophyllum* was mainly built of β(1→6)-D-galactose and/or β(1→2)-D-mannose units with branching points formed by α(1→3) and/or α(1→4)-L-fucose, α(1→4)-D-glucuronic acid, terminal β-D-xylose, and sometimes α(1→4)-D-glucose. Sulfate groups on the fucans are located at C-4 of a (1→3)-linked unit or C-2 of a (1→4)-linked residue (Fig. 2.3; Duarte et al., 2001). Galactose is found in trace amounts in the fucans from *Himanthalia lorea* and *Macrocystis pyrifera* (Mian and Percival, 1973) and has been reported as a major constituent, but the polysaccharides also had substantial quantities of fucose, xylose, and glucuronic acid (Percival and Young, 1974). A sulfated β-D-galactan isolated from *L. angustata* in trace amounts was considered a fucoidan with β-D-galactose as the major sugar and L-fucose and D-glucuronic acid as trace components (Nishino et al., 1994).

2.1.2 Anti-tumor bioactivity of FCSPs

FCSPs in brown seaweed have been subject to many scientific studies due to their diverse biological functions including anti-tumor and immunomodulatory activities (Alekseyenko et al., 2007; Maruyama et al., 2006). There are very few published reports on the relationship between chemical properties and anti-tumor activity of FCSPs. (Li et al., 2008; Alekseyenko et al., 2007; Koyanagi et al., 2003). However, it was suggested that the bioactive properties of FCSPs are mainly determined by the fucose sulfated chains (Nishino et al., 1994; Mourão et al., 1996); nevertheless, the anti-cancer activity of FCSPs was recently revealed to not be a function of a single but a combination of many factors such as the amount of sulfate groups, monosaccharide residues ratio, and the linkage type of the sugar residues (Ermakova et al., 2011; Ale et al., 2011). The available findings indicate that the anti-tumor activity of FCSPs may be associated with a significant enhancement of the cytolytic activity of NK cells augmented by increased production of macrophage-mediated immune response-signaling molecules (Maruyama et al., 2003; Takahashi et al., 1983; Teruya et al., 2009), namely interleukin (IL)-2, interferon (IFN)-γ, and IL-12 (Ale et al., 2011; Maruyama et al., 2003), and induction of apoptosis (Ale et al., 2011).

Macrophage activation by polysaccharides is mediated through specific membrane receptors. The major receptors reported for polysaccharide recognition in macrophages are glycoproteins including Toll-like receptor-4 (TLR-4), cluster of differentiation 14 (CD14), competent receptor-3 (CR-3), and scavenging receptor (SR) (Teruya et al., 2009). Activation of these receptors is mediated by intracellular signaling pathways, and the family of mitogen-activated protein kinases (MAPKs) plays a

critical role, notably in the production of nitric oxide (NO), which can lyse tumors (Teruya et al., 2009). MAPK family members such as p38 MAPK, extracellularly regulated kinase (EKR1/2), and stress-activated protein kinase/c-Jun-N-terminal kinase play an important role in the activation of macrophages by polysaccharides such as FCSPs (Teruya et al., 2009; Aisa et al., 2005)(Fig. 2.4). Activated MAPKs lead to activation of transcription factors resulting in induction of various genes (Teruya et al., 2009). Activation of macrophages induces the production of cytokines such as interleukin-12 (IL-12) which in turn stimulate the development of T-cells (Fig. 2.4). T-cells produce interleukin-2 (IL-2) that in turn activates NK cells proliferation. The NK cells themselves produce immunologically important cytokines, notably IFN-γ, which can further provoke the participation of macrophages in the stimulation of T-cell via induction of IL-12 (Maruyama et al., 2006; Teruya et al., 2009) (Fig. 2.4).

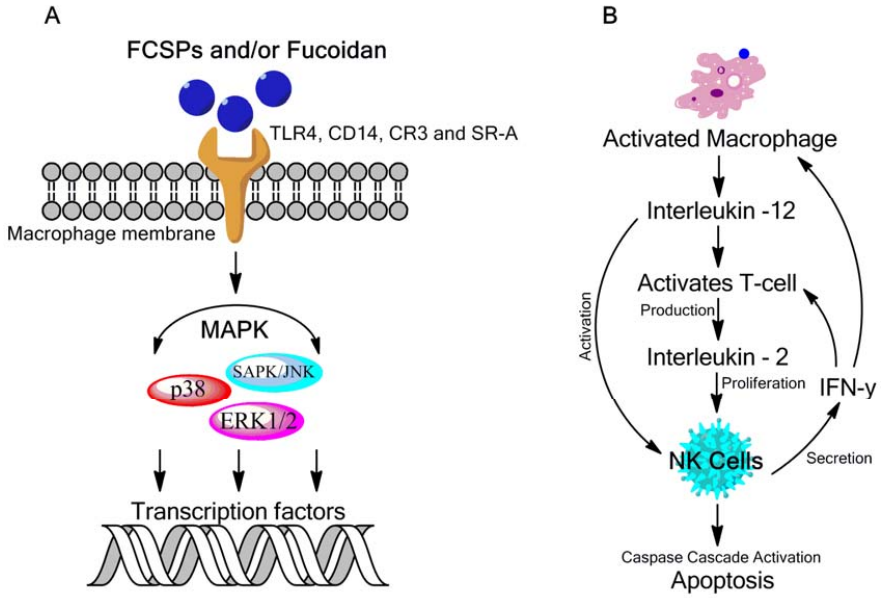

Fig 2.4. Proposed mechanism responsible for fucoidan bioactivity: (a) Macrophage activation by fucose-containing sulfated polysaccharides (FCSPs) mediated through specific membrane receptor activation, namely Toll-like receptor (TLR)-4, cluster of differentiation 14 (CD14), competent receptor-3 (CR-3), and scavenging receptor (SR), which in turn induce intracellular signaling via mitogen-activated protein kinases (MAPKs); (b) activation of macrophages lead to production of cytokines such as interleukin (IL)-12, IL-2, and interferon (IFN)-γ, which enhances NK cell activation that may further stimulate T cell activation via IFN-γ.

The anti-tumor mechanism of FCSPs appears to be associated with the significant enhancement of the cytolytic activity of NK cells augmented by increased production of the macrophage-mediated immune response, namely IL-2, type II IFN-γ, and IL-12 (Maruyama et al., 2003; Maruyama et al., 2006). NK cells are large granular lymphocytes that are found throughout the body that contain cytotoxic substances which are important for the protection against some tumors. The slated NK cell killing occurs via release of granules containing perforin, which effectively opens up pores in target cell membranes through which the granzymes can enter and induce apoptosis (Kindt et al., 2007; Lydyard et al., 2000). FCSPs was reported to induce apoptosis in HT-29 colon cancer cells (Kim et al., 2010), MCF-7 human breast cancer cells (Yamasaki-Miyamoto et al., 2009), and HS-Sultan human lymphoma cells (Aisa et al., 2005). The importance of apoptosis as a killing mechanism used by the immune system is that targeted cells can be rapidly removed by phagocytes without induction of an inflammatory response.

IL-2, which is made by T cells, is a critical autocrine growth factor that is required for proliferation of T cells and NK cells (Lydyard et al., 2000). NK cell secretion of type II IFN-γ activates macrophages, inducing IL-12 secretion, activating NK cells, and creating a system of positive feedback that increases the activation of both cell types within an infected cell or tissue (Parham 2009; Kindt et al., 2007). Hence, it was suggested that stimulation with IL-2 and IL-12 promotes IFN-γ secretion by NK cells, probably due to enhancing NK cell activity by FCSPs (Maruyama et al., 2006). IL-12 stimulation alone was reported to produce only moderate augmentation of NK cell cytotoxicity. However, it increases the catalytic activity of lymphocytes against autologous targets in synergy with IL-2 (Nastala et al., 1994).

2.2 FCSPs extraction and chemical composition: past and present

Several extraction and purification procedures have been used for many years to isolate fucoidan from brown seaweed. Extraction using dilute acetic acid and subsequent purification was first performed by Kylin in 1913 to isolate the substance from various species of Laminaria and Fucus (Kylin, 1913). Kylin reported that fucoidan extracted in this way mainly contained fucose and also observed that the fucose occurred together with mannitol, alginic acid, and laminarin (Kylin, 1913) (Table 1); we now know that this interpretation was a result of co-extraction of these latter contaminants with fucoidan. Two years later, Kylin reported that fucoidan isolated from *L. digitata* contained methylpentose, interpreted as L-fucose, as well as some other pentoses (Kylin, 1915). A

parallel report was published that year by Hoagland and Lieb (1915), who isolated another water-soluble polysaccharide that was closely related to, if not identical with, fucoidan from *Macrocystis pyrifera* and was shown to contain L-fucose and a high proportion of calcium and sulfate (Table 1). Hoagland and Lieb (1915) did not compare their extraction using Na_2CO_3 soaking to one without, but their report is nevertheless the first example of how the extraction procedure may influence purity and, in turn, the analyzed composition of the extracted FCSPs (Table 1). Bird and Haas (1931) used fresh *L. digitata* fronds to obtain fucoidan by soaking them in water and precipitating crude sulfate from the extract using ethanol. Uronic acid was also present in this preparation (Table 1).

Table 1. Historic view of very early work, 1913–1950s, of fucoidan or fucose-containing sulfated polysaccharide extraction and their composition from different brown seaweed species.

Year	Brown Seaweed sp.	Reported FCSPs Composition	Extraction Method	References
1913	*Laminaria* and *Fucus*	Fucoidan contaminated with mannitol, alginate, and laminarin	Dilute acetic acid extraction	Kylin, 1913
1915	*Laminaria digitata*	Fucoidan contained methylpentose, L-fucose, and some pentoses	Dilute acetic acid extraction	Kylin, 1915
1915	*Macrocystis pyrifera*	Dominantly alginic acid with fucose-sulfate	Seaweed was soaked in 2% Na_2CO_3 for 24 h, filtered, combined with HCl, and the resulting precipitate was filtered and then finally resolved in 2% Na_2CO_3	Hoagland and Lieb, 1915
1931	*L. digitata*	Substantial amounts of calcium sulfate and uronic acid	Soaking of seaweed in water, precipitation of crude sulfate by ethanol	Bird and Haas, 1931
1931	*Macrocystis pyrifera*	Methylpentose monosulphate monosulfate polymer with mainly fucose and alginate contaminants	Repeated extraction with 2% HCl at room temperature for 48 h, precipitated with 90% EtOH	Nelson and Cretcher, 1931
1937	*L. digitata*	Sulfate residue must be substituted by fucose or another sugar residue	Prepared by precipitating the droplets exuded from seaweed in boiling ethanol	Lunde et al., 1937
1950	*Fucus vesiculosus*, *Fucus spirales*, *Himanthalia lorea*, *Laminaria clustoni*	Substantial amount of fucose and sulfate; and small amounts of uronic acid, galactose and xylose; ash was mainly calcium sulfate	Acid pH 2–2.5, 70°C, 1 h, 3 times; or aqueous, 100°C, for 24 h, lead acetate treatment, barium hydroxide added	Percival & Ross, 1950
1952	*F. vesiculosus*	Fucose, ash, sulfate	pH 2–2.5, 70°C, 1 h, 3 times	Black et. al., 1952

The early FCSPs extraction procedures involved dilute acid treatment either with acetic or hydrochloric acid as the first extraction step with the purpose of hydrolyzing the non-FCSP polysaccharides (Table 1). However, the extraction and purification methods employed in different studies to isolate fucoidan/FCSPs from brown seaweed biomass have been modified to different extents since the first reports in 1913 and 1915. For example, Nelson and Cretcher (1931) extracted fucoidan from *Macrocystis pyrifera* by repeated extended (48-h) extraction with dilute HCl followed by FCSP isolation by ethanol precipitation and revealed the presence of sulfate in the form of ester groupings in the precipitated product. They also confirmed that fucose was the only sugar identified in the unhydrolyzed residue after acid hydrolysis, even though their product contained uronic acid, which was considered to be due to alginate contamination (Table 1).

2.2.1 FCSPs composition studies, 1930–1950

The studies by Nelson and Cretcher (1931) revealed the presence of sulfate in the form of ester groupings and confirmed that fucose was the only sugar identified after hydrolysis, although their product contained uronic acid, which was considered to be due to alginic acid contamination (Table 1). Please note the terminology used is quite confusing; nevertheless, with recent advances in chemical analyses, we now know that alginic acid and alginate comprise guluronic and mannuronic acids. Our understanding today that alginic acid, or alginate, is a linear hydrocolloid polymer that consist of blocks of (1→4)-linked β-D-mannuronate and its α-L-guluronate residues, that these 2 monomers are C-5 epimers, and that the detailed structure of alginate may have mannuronate and guluronate in homopolymeric blocks of consecutive mannuronate residues (M-blocks), consecutive guluronate residues (G-blocks), or in structural units of alternating mannuronate and guluronate residues (MG-blocks). Uronic acids were determined to cover several different structures. This work addressed the significance of the differences and introduced consistent terminology by grouping these as uronic acids, especially since they do not belong to the FCSPs. Later, Lunde et al. (1937) prepared fucoidan by directly precipitating the droplets exuded from freshly gathered *L. digitata* fronds in ethanol (Table 1). After purification via product precipitation using boiling ethanol, they obtained a FCSP specimen that contained 33–37% methylpentose (interpreted correctly as fucose) and 26–30% ash in which the sulfate content was 17–19%, which made up approximately half of the total estimated sulfate in the polysaccharide (35–38%). They proposed a structural unit formula, (R-R'-O-SO_2-OM)n, for fucoidan and suggested that R was fucose or another pentose sugar residue, R' was unknown, and M was Na+, K+, (½)Ca^{2+}, or (½)Mg^{2+} (Lunde et al., 1937) (Table 1).

Specimens from *F. vesiculosus*, *F. spirales*, *H. lorea,* and *L. clustoni* were prepared by Percival and Ross (1950) for FCSPs extraction using boiling water for 24 h, and the alginates and protein were removed with lead acetate and the addition of barium hydroxide. The resulting lead hydroxide-fucoidan complex was then decomposed using dilute sulfuric acid and the fucoidan was isolated after prolonged hydrolysis and filtration. The purest specimen was from *H. lorea,* which contained the following: a substantial amount of fucose and sulfate; small quantities of uronic acid, galactose, and xylose; metals; and ash, which was mainly calcium sulfate. These workers believed that the principal constituent of fucoidan is a polyfucose monosulfate and that other constituents arise from impurities (Table 1). A parallel work was done by Conchie and Percival (1950) in which fucoidan from *Fucus vesiculosus* was methylated. It was believed that the main residue in fucoidan was 1→2 linked of L-fucopyranose units carrying a sulfate group in C-4 (Conchie and Percival 1950). However, we now know that this is not the case because advanced analyses have verified that the backbone of fucoidan from *F. vesiculosus* consists of alternating α(1→3) and α(1→4) linkages of L-fucopyranose residues (Fig. 2.2).

2.2.2 Lab-scale extraction of fucoidan, 1950s

In the pursuit of obtaining extensive quantities, a laboratory-scale extraction of fucoidan was performed by Black et al. (1952). Interestingly, they referred to the extracted product as a "polyfucose ethereal sulfate occurring in the Phaeophyceae." Their optimal fucoidan extraction procedure was as follows: one part by weight of dried ground seaweed and 10 parts by volume of 0.1 M hydrochloric acid at pH 2.0–2.5 contacted at 70°C with constant stirring for 1 h. A single acid hydrolysis extraction treatment using this method recovered about 50% by weight (w/w) of the theoretical maximum of fucoidan (recovered yield measured as % fucose obtained as % of total fucose in the seaweed DW), whereas 3 rounds of acid extraction recovered >80% of the fucose present; the triple acid hydrolysis treatment (0.1 M HCl, pH 2.0–2.5, 70°C, 1 h x 3) was therefore selected as the optimal extraction method (Table 1). After the acid hydrolysis treatment the crude fucoidan was isolated by fractional precipitation with alcohol and further purified by precipitation after the addition of formaldehyde (Black et al., 1952). Using this procedure, crude fucoidan samples containing 30–36% fucose were obtained; for example, the fucoidan recovered from *F. vesiculosus* using the optimal extraction protocol was analyzed to contain (by weight) 44% fucose, 26% total sulfate, and 31% ash (Black et al., 1952). In terms of yields of percentage of total fucose, the results obtained for the 4 different algal species were: *Pelvetia canaliculata,* 76%; *F. vesiculosus,* 62%; *Ascophyllum nodosum,* 54%; and *L. cloustoni,* 20% (Black et al., 1952). The authors suggested that a more efficient extraction methodology, i.e., extracting higher fucose

yields, could be achieved by increasing the water/seaweed ratio, extraction time, or extraction number.

2.2.3 FCSPs extraction, 1970s–present

Carbohydrates of the brown seaweeds were successively extracted from *H. lorea*, *Bifurcaria bifurcata,* and *Padina pavonia* using dilute acid followed by alkaline or using a water, acid, and alkali sequence. Prior to extraction, the seaweed frond was pretreated with formaldehyde to polymerize phenolic constituents that could otherwise be contaminant to various extracts (Mian and Percival, 1973). This technique produced complex mixture of glucan, FCPSs, and alginic acid, which could be separated by fractional precipitation with ethanol, calcium salts, or cetyltrimethylammonium hydroxide, or by fractionation on resin columns. Fucoidan extracted in this fashion consisted of heteropolysaccharide comprising different proportions of fucose, glucuronic acid, xylose, and half-ester sulfate together with a trace of galactose. The result of this extraction method also showed that sulfate and uronic acid contents in the fucoidan separated from aqueous calcium chloride and acid extracts varied significantly by species.

A study was conducted on the purification of a sulfated heteropolysaccharide from *S. linifolium* to elucidate its structural components. The extraction was done using hydrochloric acid pH 1.0 for 3 h at 80°C; the extract was neutralized using aqueous sodium carbonate and precipitated with ethanol. The resulting sulfated polysaccharide material was acid-hydrolyzed and it was proposed that the backbone was composed of glucuronic acid, mannose, and galactose residues with partially sulfated side chains of galactose, xylose, and fucose residues (Abdel-Fattah et al., 1974).

Extraction using an aqueous alkali solution or dilute acid at ambient or slightly elevated temperatures has been the convenient method to produce fucoidan for many years. In recent years, pretreatment of seaweed biomass has been found advantageous to eliminate low molecular components (e.g., phenols) using a mixture of methanol, chloroform, and water (Bilan et al., 2006); removal of protein can be facilitated using proteolytic enzymes (Rocha et al., 2005). A useful procedure to transform alginate in the residual biomass into insoluble calcium salts uses aqueous calcium chloride (Bilan et al., 2002).

2.3 Seaweed products and biomass potential

The growth of seaweed production and developments of advanced technological farming indicates that the seaweed industry is progressively evolving and thus paves the way to new application opportunities (Buck and Buchholz, 2004). For many years, seaweed has been utilized as a source of

dietary nourishment among Asian constituents. In recent years, more applications have used seaweed resources because of their unique characteristics and properties. Seaweed has been proposed as a source of important compound that can be incorporated in the production of functional food ingredients, pharmaceuticals, and cosmetics (Bodin- Dubigeon et al., 1997; Bixler, 1996; De Roeck-Holtzhauer, 1991). Gel-forming polysaccharides such as carrageenan and alginate are mostly known and commercially important seaweed products. Production of alternative fuels from non-starch biomass has recently directed the attention to utilization of marine macroalgae as sources of biomass for biofuel production (Knauf and Moniruzzaman, 2004). Moreover, healthy and bioactive components, notably the FCSPs found in brown seaweed biomass, have also become subject of much research and product development.

2.3.1 Phycocolloids

The global market for phycocolloids such as agar, carrageenans, and alginates is estimated to be worth annually $585 million US (McHugh, 2003). To date, the red seaweed has been harvested for food consumption in some regions of Asia, but many of these areas have engaged in farming red seaweed that is intended for the production of agar and carrageenan, while brown seaweed is most harvested naturally for alginate production (Crawford, 2002).

Carrageenan is composed of a linear galactose backbone with varying degrees of sulfation (15–40%) and are mainly composed of disaccharide repeating units of an α-(1→4)-linked D-galactopyranose or 3,6-anhydro-D-galactopyranose residue and a β-(1→3)-linked D-galactopyranose. The sulfated groups are covalently attached to individual galactose residues via ester linkages to the carbon atoms C-2, C-4, or C-6 (De Ruiter and Rudolph, 1997). Carrageenan is applied as a stabilizing agent to many food products and other industrial and pharmaceutical applications (van de Velde and De Ruiter, 2002). Among the leading species of red algae responsible for most of today's commercial carrageenan production are *K. alvarezii* and *E. denticulatum*.

In addition, alginate another gel-forming sulfated polysaccharide extracted from brown seaweed has been utilized for various applications including foods and feeds, pharma/medical, and industrial preparations. Alginate is composed of mannuronic (M) and guluronic (G) acids with (1, 4) linkages, and its structure varies according to the monomer position on the chain, forming either homopolymeric (MM or GG) or heteropolymeric (MG or GM) segments (Percival and McDowell, 1967). The molecular weight of alginate is generally 500–1000kDa. Its solubility is influenced by factors such as pH, concentration, ions in solution, and the presence of divalent ions such as calcium (Morris and Norton, 1983).

2.3.2 Seaweed biomass for bioenergy production

Only few seaweed species have been exploited for commercial production (i.e., red and brown seaweed); hence, this study opens the way for other species to provide a basis for additional potential applications of seaweed biomass, namely bioenergy production. The cultivation of seaweed poses advantages to terrestrial crops since they have high growth rates and can be continuously harvested (Rasmussen et al., 2009). The estimated annual production of *U. lactuca* per hectare was 45 tons DW, which is 3× greater the yield of conventional food or energy crops (Bruhn et al., 2011). However, the production is believed to be significantly higher once growth conditions (exploiting flue gas as a source of carbon) and nutrients (fishery effluents as a source of nitrogen) are optimized. A study on the anaerobic digestion of sea lettuce (*Ulva* sp.) suggested that the methane gas yield from washed and grinded sea lettuce biomass is about 180 mL g^{-1} VS (volatile solid-based), while that from non-pretreated biomass is about 70% (Otsuka and Yoshino, 2004). Biogas production from fresh and macerated *U. lactuca* yielded up to 271 mL CH_4 g^{-1} VS, which is in the range of the methane production from cattle manure and land-based energy crops such as grass clover. Drying of the biomass resulted in a 5–9-fold increase in weight-specific methane production compared to wet biomass (Bruhn et al., 2011).

2.4 Seaweed production

Seaweed has been traditionally cultured for centuries in several Asian countries including China, Japan, and Korea (Crawford, 2002). Most seaweed production came from the harvest of wild stocks in these countries, although limited culture had been established in countries such as the Philippines and Indonesia (Trono, 1990). The species cultivated include *Kappaphycus* and *Eucheuma*. The leading seaweed exporter, the Philippines, increased production from 675 tons in 1967 to 65,617 tons in 2009 and 80,000 tons in 2010, but the country is still importing raw seaweed materials from Indonesia (www.siap.com.ph, 2011). The world demand for seaweed in 2003 was 220,000 tons for *K. alvarezii* and *E. denticulatum* with an expected 10% annual increase in demand (Sievanen et al., 2005). Indonesia, on the other hand, raised its production from 1,000 million tons (MT) in 1966 to approximately 27,874 tons in 2001 and estimated 10 MT in 2015 (Sievanen et al., 2005). The production levels of algae in 2002 reached 18.6 MT (FAO, 2004). Europe had only a 6.3% share of the global world production of brown algae (362,000 tons FW) and about 0.3% of red algae (9,400 tons FW) with <200 tons of macroalgae produced in aquaculture in 2002 (FAO, 2004).

Several sea-based cultivation design methods have been developed and tested. The most commonly used was a fixed off-bottom long line, hanging long line, and combination of the 2 (Hurtado et al.,

2001). System off-shore designs for *Laminaria* culture tested within the area of Helgoland farm, Germany (Buck and Buchholz, 2004) used longline, ladder (tandem longline), and ring-shaped designs for attachment of algae-seeded culture lines. The off-shore cultivation ring developed by Buch and Buchholz (2004) proved resistant to rough weather conditions (6-m wave heights) and current velocities (2 m s^{-1}) and also permitted easy handling. About 75% of the cultivation ring culture lines were fully covered by *L. saccharina*, with a total fresh weight of 304 kg after a 6-month grow-out phase in the sea. Harvesting of the ring construction is performed by towing it to the shore and lifting it by cranes, or harvest at sea can be performed using boat-based cranes. However, the cost of a fully mounted ring is quite high (€ 1000) as suggested by Buch and Buchholz (2004).

On the other hand, the nuisance seaweed *U. lactuca* has been shown to be suitable for propagation under controlled conditions (Lee, 2000). For this reason, cultivation of *U. lactuca* in tanks for either crude biomass production for bioenergy or production of biologically active compounds is receiving increased attention. The green algae *Ulva* sp. is cultivated in tanks in Denmark for bioenergy production (Rasmussen et al., 2009). The production of *Ulva* in Denmark is estimated to be an average of 45 tons DW/ha per annum (200 days of sufficient light conditions). Critical consideration when cultivating *Ulva* spp. in tanks is the frequently occurring sporulation events result in the loss of algal tissue (Werner et al., 2003).

2.5 Paper 1: Important determinants for fucoidan bioactivity: a critical review of structure-function correlations and extraction methods for FCSPs from brown seaweeds

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Review

Important Determinants for Fucoidan Bioactivity: A Critical Review of Structure-Function Relations and Extraction Methods for Fucose-Containing Sulfated Polysaccharides from Brown Seaweeds

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Abstract: Seaweeds—or marine macroalgae—notably brown seaweeds in the class Phaeophyceae, contain fucoidan. Fucoidan designates a group of certain fucose-containing sulfated polysaccharides (FCSPs) that have a backbone built of (1→3)-linked α-L-fucopyranosyl or of alternating (1→3)- and (1→4)-linked α-L-fucopyranosyl residues, but also include sulfated galactofucans with backbones built of (1→6)-β-D-galacto- and/or (1→2)-β-D-mannopyranosyl units with fucose or fuco-oligosaccharide branching, and/or glucuronic acid, xylose or glucose substitutions. These FCSPs offer several potentially beneficial bioactive functions for humans. The bioactive properties may vary depending on the source of seaweed, the compositional and structural traits, the content (charge density), distribution, and bonding of the sulfate substitutions, and the purity of the FCSP product. The preservation of the structural integrity of the FCSP molecules essentially depends on the extraction methodology which has a crucial, but partly overlooked, significance for obtaining the relevant structural features required for specific biological activities and for elucidating structure-function relations. The aim of this review is to provide information on the most recent developments in the chemistry of fucoidan/FCSPs emphasizing the significance of different extraction techniques for the structural composition and biological activity with particular focus on sulfate groups.

Keywords: fucoidan; antitumor; anticoagulant; extraction; sulfated polysaccharides

1. Introduction

Fucoidan is a term used for a class of sulfated, fucose rich, polysaccharides found in the fibrillar cell walls and intercellular spaces of brown seaweeds of the class Phaeophyceae. These fucose-containing sulfated polysaccharides (FCSPs) principally consist of a backbone of (1→3)- and (1→4)-linked α-L-fucopyranose residues, that may be organized in stretches of (1→3)-α-fucan or of alternating α(1→3)- and α(1→4)-bonded L-fucopyranose residues. The L-fucopyranose residues may be substituted with sulfate (SO_3^-) on the C-2 or C-4 (rarely on C-3), with single L-fucosyl residues and/or with short fucoside (fuco-oligosaccharide) side chains. If present, the fucoside side chains are usually O-4 linked to the α-L-fucopyranose backbone residues. However, as FCSPs structures of more brown seaweeds are being elucidated, as discussed further in the present review, it appears that FCSPs cover a broader range of complex polysaccharides than only those having fucan backbones. Apart from variations in the sulfate content and substitutions, also the monosaccharide composition of FCSPs varies among different species of brown seaweeds. Hence, in addition to fucose, different types of FCSPs may also contain galactose, mannose, xylose, glucose and/or glucuronic acid—usually in minor amounts [1].

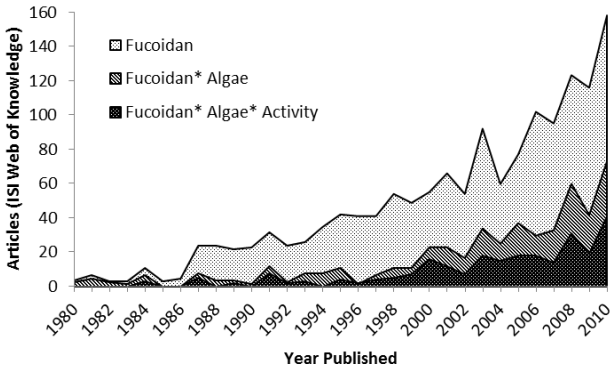
According to the ISI Web of Knowledge (Thomson Reuters) the number of published articles with the topic assigned as "fucoidan" has increased significantly since fucoidan, or "fucoidin" as it was first called, was first isolated from brown algae in 1913 [2]; in particular, a profound increase in the number of papers has taken place during the last 5–10 years. By now, the published papers related to fucoidan hit approximately 1800 (August 2011, Figure 1). The recent interest has mainly focused on the potentially beneficial biological activities of fucoidan and FCSPs in humans including antitumor, immunomodulatory, anti-inflammatory, antiviral, antithrombotic, anticoagulant, and antioxidant effects as well as specific activities against kidney, liver and urinary system disorders.

While the development of research efforts involving FCSPs and their potential applications are advancing, the understanding of the mechanisms and the particular structural features of the FCSPs being responsible for the various biological activities is still incomplete. Seaweeds, including various brown seaweeds such as *Undaria* and *Laminaria* spp., are part of the food culture in Asia, notably in Japan, the Philippines, and Korea, and seaweed extracts have also been used as a remedy in traditional medicine. However, no standardized FCSPs extraction or purification protocols exist, and no specific pharmaceutical, dermatological, or nutraceutical applications have as yet been officially approved for these polysaccharides or their lower molar mass oligosaccharide derivatives.

It is our proposition that more focus should be directed to the extraction and purification processes in order to obtain consistent protocols that account for the biodiversity of FCSPs from different seaweeds and to retain the structural features of significance for the specific bioactivity properties of FCSPs. The development and use of such consistent extraction procedures would also help in achieving a better understanding of structure-function relationships of FCSPs. The aim of this review is to bring attention to the detailed structural features of FCSPs in relation to their marine algal sources

and the extraction methodology, and to highlight recent knowledge concerning the structural determinants for FCSPs bioactivity.

Figure 1. The trend during three decades of research on fucoidan as depicted by the number of published articles (Thomson Reuters, ISI Web of Knowledge). The number of articles was obtained according to topics being assigned in the ISI Web of Knowledge search engine with the following topic search terms: Fucoidan; Fucoidan*Algae; Fucoidan*Algae*Activity.



2. Historic Overview: FCSPs Extraction Procedures and Chemical Analyses

Extraction using dilute acetic acid followed by purification was used by Kylin already in 1913 to isolate "fucoidin", subsequently referred to as fucoidan, from various species of *Laminaria* and *Fucus* [2]. Already in this first seminal report, Kylin reported that fucoidan extracted in this way mainly contained fucose, but also observed that the fucose occurred together with mannitol, alginic acid and laminarin [2] (Table 1); we now know that this interpretation was a result of co-extraction of these latter contaminants with the fucoidan. Two years later, Kylin reported that fucoidan isolated from *Laminaria digitata* contained methylpentose, interpreted as L-fucose, as well as some other pentoses [3]. A parallel report was published that same year by Hoagland and Lieb (1915) [4] who isolated a water-soluble polysaccharide from *Macrocystis pyrifera* that was closely related to if not identical with "fucoidan", and shown to contain L-fucose as well as relatively high levels of calcium and sulfate. They employed a Na_2CO_3 soaking step and addition of hydrochloric acid which is why they also—if not mainly—isolated alginic acid (or alginate) during the extraction (Table 1). The rationale behind the extraction of alginate from the seaweed with Na_2CO_3 soaking is to convert all the alginate salts, typically calcium and magnesium alginate, to the sodium salt. Please note that the terminology used is confusing; alginic acid or alginate does not designate one particular monosaccharide or one type of homo-polysaccharide. With the advances in chemical analyses it is now known that alginic acid or alginate is comprised of guluronic and mannuronic acids; which are C-5 epimers. Structurally, alginic acid, or alginate, is a linear hydrocolloid polymer that consists of blocks of (1→4) linked β-D-mannuronate and α-L-guluronate residues. The detailed structure of alginate may have mannuronate and guluronate in homopolymeric blocks of consecutive mannuronate residues

(M-blocks), in consecutive guluronate residues (G-blocks), or in structural units of alternating mannuronate and α-L-guluronate residues (MG-blocks). Both mannuronic acid and α-L-guluronic acids are uronic acids, and they have in the past been analytically determined as uronic acids. However, uronic acids also include several other structures, e.g., glucuronic and galacturonic acids. In this review, we will address the significance of the compositional and structural differences, but also attempt to introduce a consistent terminology by grouping these compounds as uronic acids, especially as homopolymers do not belong to the group of FCSPs. Hoagland and Lieb (1915) [4] did not compare their extraction with Na_2CO_3 soaking to one without, but their report is nevertheless the first example of how the extraction procedure may influence the purity and, in turn, the analyzed composition of the extracted FCSPs (see Table 1).

Table 1. Historic view of very early work, from 1913–1950, of fucoidan or FCSPs extraction and their reported composition from different brown seaweed species.

Year	Brown seaweed sp.	Reported FCSPs composition	Extraction method	References
1913	*Laminaria and Fucus*	Fucoidan contains fucose, that occurs together with mannitol, alginate and laminaran	Dilute acetic acid extraction	Kylin, 1913 [2]
1915	*Laminaria digitata*	Fucoidan contains L-fucose and other pentoses	Dilute acetic acid extraction	Kylin, 1915 [3]
1915	*Macrocystis pyrifera*	Mainly alginic acid, with some fucose-sulfate	Soaking in 2% Na_2CO_3 for 24 h, filtration, HCl addition, recovery of precipitate by filtration, redissolution in 2% Na_2CO_3	Hoagland and Lieb, 1915 [4]
1931	*Laminaria digitata*	Substantial amounts of calcium sulfate and uronic acid	Soaking of the seaweed in water, precipitation of crude, sulfated polysaccharides by ethanol	Bird and Haas, 1931 [5]
1931	*Macrocystis pyrifera*	Methylpentose monosulphate polymer with fucose and alginate contaminants	Repeated extraction with 2% HCl at room temperature for 48 h, precipitated with 90% ethanol	Nelson and Cretcher, 1931 [6]
1937	*Laminaria digitata*	Proposed $(R\text{-}R'\text{-}O\text{-}SO_2\text{-}OM)_n$ with R = fucose, R′ as unknown, M being Na, K, $Ca_{0.5}$, or $Mg_{0.5}$	Precipitation of droplets exuded from seaweed in boiling ethanol	Lunde *et al.*, 1937 [7]
1950	*Fucus vesiculosus*, *Fucus spirales*, *Himanthalia lorea*, *Laminaria clustoni*	Substantial amounts of fucose and sulfate; small amounts of uronic acid, galactose and xylose; metals and ash were also detected, ash was mainly calcium sulfate	Aqueous extraction at ~100 °C for 24 h, extract treated with lead acetate (to precipitate alginate and proteins), filtrate solution treated with $Ba(OH)_2$ to precipitate a "hydroxide-fucoidin complex"	Percival and Ross, 1950 [8]
1952	*Fucus vesiculosus*	Fucose, ash, sulfate	0.1 M HCl at pH 2–2.5 and 70 °C for 1 h, 3-times, fractional precipitation with ethanol	Black *et. al.*, 1952 [9]

The early FCSPs extraction procedures were based on using a dilute acid treatment, with either acetic or hydrochloric acid used as a first "extraction" step with the purpose of hydrolyzing the non-FCSP polysaccharides (Table 1). However, the extraction and purification methodologies employed in

different studies to isolate fucoidan/FCSPs from brown seaweed biomass have been modified to different extents since the first reports from 1913 and 1915. Bird and Haas (1931) [5], for example, used soaking of the brown alga biomass in water and precipitation of crude sulfate from the extract with ethanol to obtain fucoidan from *L. digitata* (Table 1). The product of this extraction was also found to contain relatively high levels of ash. The high ash levels were presumably chiefly a result of the presence of calcium sulfate in the algal polysaccharides. For the compositional analysis, acid hydrolysis using H_2SO_4 was typically used. This acid hydrolysis step might by itself have contributed a substantial amount of sulfate. As a consequence, the use of H_2SO_4 clearly biased the interpretation of the compositional analysis. Hence, the fucoidan isolated by Bird and Haas (1931) [5] was designated as carbohydrate sulfate (*i.e.*, containing esterified sulfate) since the total sulfate content was approximately the double of that found in the ash. Uronic acid was also present in the FCSPs preparation extracted from *L. digitata* [5] (Table 1).

Nelson and Cretcher (1931) [6] extracted fucoidan from *Macrocystis pyrifera* by repeated, extended (48 h) extraction with dilute HCl followed by isolation of the FCSPs by ethanol precipitation, and revealed the presence of sulfate in the form of ester groups in the precipitated product. They also confirmed that fucose was the only sugar identified in the unhydrolyzed residue after acid hydrolysis, even though their product contained uronic acid, considered to be due to alginate contamination (Table 1). Later, Lunde *et al.* [7] prepared fucoidan by directly precipitating the droplets exuded from freshly gathered *L. digitata* fronds in ethanol (Table 1). After purification via precipitation of the product from boiling ethanol they obtained a FCSPs specimen that contained 33–37% methylpentose (interpreted correctly as fucose), and 26–30% ash in which the sulfate content was 17–19%, which made up approximately half of the total sulfate estimated in the polysaccharide (35–38%). They proposed a structural unit formula, $(R\text{-}R'\text{-}O\text{-}SO_2\text{-}OM)_n$, for fucoidan and suggested that R was fucose or another pentose sugar residue, R′ was unknown, and M was Na^+, K^+, $(½)Ca^{2+}$ or $(½)Mg^{2+}$ [7] (Table 1).

"In an attempt to reconcile some of the conflicting views on the nature of fucoidin" crude fucoidan extracts from *F. vesiculosus*, *F. spirales*, *Himanthalia lorea* and *Laminaria clustoni* were prepared by Percival and Ross (1950) [8]. Their methodology involved boiling of the seaweed biomass in neat boiling water for 24 h (hydrolysis treatment) followed by removal of alginates and protein by addition of lead acetate, then, after addition of barium hydroxide (presumably to precipitate alginate) the fucoidan was isolated as a crude lead hydroxide complex (Table 1). In order to isolate lead free fucoidan, the lead hydroxide complex was treated with dilute H_2SO_4 and fucoidan was then isolated after prolonged dialysis and filtration. The purest fucoidan specimen obtained was from *H. lorea*. This fucoidan isolated from *H. lorea* contained substantial amounts of fucose and sulfate; as well as small quantities of uronic acid, galactose and xylose. Metals and ash were also detected, and the ash was mainly made up of calcium sulfate [8]. Based on the data obtained the authors believed that the principal constituent of fucoidan was a polyfucose with one sulfate substitution on each fucose residue and that other constituents arose from adventitious impurities. A parallel paper was published in which it was proposed that the core structure of fucoidan from *F. vesiculosus* was built of 1→2 linked L-fucopyranose units, each carrying a sulfate group on C-4 [10]. As discussed later, the interpretation that the fucosyl units in the fucoidan were 1→2 linked turned out to be incorrect, as more advanced

analyses have now verified that the backbone of fucoidan from *F. vesiculosus* consists of alternating α(1→3) and α(1→4) linkages [11].

In the pursuit to obtain extensive quantities, a laboratory-scale extraction of fucoidan was reported by Black *et al.* [9]. Interestingly, the extracted product was referred to as a "polyfucose ethereal sulphate occurring in the *Phaeophyceae*". Their optimal fucoidan extraction procedure was as follows: One part by weight of dried ground seaweed and 10 parts by volume of 0.1 M hydrochloric acid at pH 2.0–2.5 contacted at 70 °C with constant stirring for 1 h. A single acid hydrolysis extraction treatment using this method recovered about 50% by weight (w/w) of the theoretical maximum of fucoidan (recovered yield measured as % fucose obtained as % of total fucose in the seaweed dry weight), whereas three rounds of the acid extraction recovered more than 80% of the fucose present; the triple acid hydrolysis treatment (0.1 M HCl, pH 2.0–2.5, 70 °C, 1 h × 3) was therefore selected as the optimal extraction method (Table 1). After the acid hydrolysis treatment the crude fucoidan was isolated by fractional precipitation with alcohol and further purified by precipitation after addition of formaldehyde [9]. By this procedure samples of crude fucoidan containing 30–36% fucose were obtained; for example, the fucoidan recovered from *F. vesiculosus* using the optimal extraction protocol was analyzed to contain (by weight) 44% fucose; 26% total sulfate, and 31% ash [9]. In terms of yields, calculated as fucose as % of total fucose, the results obtained for the four different algal species were: *Pelvetia canaliculata* 76%; *F. vesiculosus* 62%; *Ascophyllum nodusum* 54%, and *L. cloustoni* 20% [9]. The authors suggested that a more efficient extraction methodology, *i.e.*, extracting higher fucose yields, could be achieved by increasing the water/seaweed ratio, extraction time or number of extractions.

In a study about 20 years later, FCSPs from the brown seaweeds *Himanthalia lorea*, *Bifurcaria bifurcata* and *Padina pavonia* were extracted successively using dilute acid, followed by alkaline or neat water extraction, acid, and alkali in sequence [12] (Table 2). Prior to extraction, the seaweed fronds were treated with formaldehyde to polymerize phenolic constituents which might otherwise contaminate the extracted saccharides [12]. This extraction protocol produced a complex mixture of glucans, fucose-containing polysaccharides, and alginic acid which could be separated by fractional precipitation with ethanol, calcium salts ($CaCl_2$) or by fractionation on resin columns. The FCSPs extracted in this fashion were reported to be heteropolysaccharides containing different levels of fucose, glucuronic acid, xylose, and esterified sulfate, together with traces of galactose [12]. The results also showed that the sulfate and uronic acid contents in the FCSPs separated from the aqueous calcium chloride and acid extracts varied significantly according to the seaweed species [12].

Another study was conducted on the purification of a sulfated heteropolysaccharide substance from *Sargassum linifolium* to elucidate its structural components [13]. The extraction was done using hydrochloric acid at pH 1.0 for 3 h at 80 °C (Table 2); the extract was then neutralized with aqueous sodium carbonate and precipitated with ethanol [13]. The resulting sulfated polysaccharide material, termed "sargassan", was proposed to be built of glucuronic acid, mannose, and galactose residues with partially sulfated side-chains composed of galactose, xylose and fucose residues [13]. As discussed later, we now know that *Sargassum* spp. do indeed contain highly complex FCSPs structures built from this array of monosaccharides.

Table 2. Extraction methods and reported chemical composition of different brown seaweed species and their corresponding order.

Species	Order	Extraction method	Composition	Reference
Cladosiphon okamuranus	Chordariales	Seaweed-H_2O suspension was treated with 30% HCl (pH 3) at 100 °C for 15 min. Supernatant was neutralized with NaOH, precipitated with $CaCl_2$ and EtOH for 20 h at 4 °C, precipitate was dissolved with H_2O then dried	fucose, glucose, uronic acid and sulfate	Nagaoka *et al.*, 1999 [14]
Adenocystis utricularis	Ectocapales	80% EtOH, 24 h, 70 °C pretreatment then extracted with water (or 2% $CaCl_2$; or HCl) for 7 h at rt, followed by exhaustive extraction at 70 °C	fucose, rhamnose, glucose, galactose, xylose, mannose, uronic acid and sulfate	Ponce *et al.*, 2003 [15]
Himanthalia lorea	Fucales	Acid + alkali + water-acid-alkali sequence in 70 °C, 4 h.	fucose, xylose, uronic acid, sulfate	Mian and Percival, 1973 [12]
Ascophyllum nodosum	Fucales	Extracted at rt and then 70 °C with 0.01 NaCl containing 1% CaCl	fucose, xylose, galactose, glucose, sulfate	Marais and Joseleau, 2001 [16]
	Fucales	Extracted with hot water and dilute alkali, formaldehyde treatment, then extracted with ammonium oxalate-oxalic acid for 6 h at 80 °C	fucose, xylose, uronic acid sulfate	Percival, 1968 [17]
Sargassum stenophyllum	Fucales	Extracted with water 7% w/v mL, 12 h, 3×. Precipitated with EtOH and $CaCl_2$ and cetylpyridinium chloride. Soluble fraction (SF) was then fractionated (F1–F6)	fucose, xylose, mannose, galactose, glucose, sulfate and uronic acid	Duarte *et al.*, 2001 [18]
Sargassum sp.	Fucales	Extracted with 0.03 M HCl at 90 °C for 4 h, single-step	Fucose, rhamnose, galactose, glucose, mannose, xylose, uronic acid, sulfate	Ale *et al.*, 2011 [19]
Sargassum linifolium	Fucales	Extracted with water at pH 1 (HCl), for 3 h at 80 °C	mannose, galactose, xylose, uronic acid and fucose residues	Abel-fattah *et al.*, 1974 [13]
Fucus evanescens; *Fucus distichus*	Fucales	Pretreatment: MeOH–$CHCl_3$–H_2O (4:2:1), then extracted 2% $CaCl_2$ for 5 h at 85 °C, precipitated and the precipitate was washed with water, stirred with 20% ethanolic solution and dissolved with water [20]	fucose, xylose, galactose, uronic acid and sulfate	Cumashi *et al.*, 2007 [21]
Fucus serratus	Fucales	Pretreatment: MeOH–$CHCl_3$–H_2O (4:2:1), then extracted 2% $CaCl_2$ for 5 h at 85 °C, the extracts were collected by centrifugation, combined, dialyzed and lyophilized [22]	fucose, xylose, mannose, glucose, galactose, uronic acid and sulfate	Cumashi *et al.*, 2007 [21]

Table 2. *Cont.*

Hizikia fusiforme	Fucales	Powdered seaweed was extracted with H_2O (1:10), 3×, 2 h at 70 °C, precipitated with EtOH and $CaCl_2$ then dried	fucose, mannose, galactose, xylose, glucose, rhamnose, arabinose, uronic acid and sulfate	Li *et al.*, 2006 [23]
Laminaria saccharina; *Laminaria digitata*; *F. vesiculosus*; *F. spiralis* *Ascophyllum nodosum*	Laminariales and Fucales	Extracted with 2% $CaCl_2$ for 5 h at 85 °C, precipitated with Cetavlon, transformation of Cetavlonic salts into calcium salts, and an alkaline treatment to remove acetyl groups and to transform fucoidan into sodium salts [24]	fucose, xylose, mannose, glucose, galactose, uronic acid and sulfate	Cumashi *et al.*, 2007 [21]
Chorda filum	Laminariales	Extracted with $CHCl_3$–MeOH–H_2O (2:4:1) followed by 80% EtOH, then extracted successively with 2% $CaCl_2$ at 20 and 70 °C, then with HCl (pH 2) and 3% Na_2CO_3, precipitated with calcium salt	fucose, xylose, mannose, glucose, galactose, uronic acid and sulfate	Chizhov *et al.*, 1999 [25]
Undaria pinnatifida	Laminariales	Ground seaweed extracted twice at rt for 6 h with 1% H_2SO_4, neutralized with 10% NaOH and lyophilized	fucose, mannose, xylose, rhamnose, galactose, glucose and sulfate	Hemmingson *et al.*, 2006 [26]
Laminaria religiosa	Laminariales	Water extraction at boiling temp. for 4 h, fucoidan fraction was obtained by using 0.09 HCl at 4 °C for 2 h, then precipitated with 85% EtOH and dried	fucose, xylose, mannose, glucose, rhamnose, uronic acid and sulfate	Maruyama and Yamamoto 1984 [27]

These early reports show that, with a few exceptions, treatment with dilute acid at ambient or slightly elevated temperature has been a preferred first step in extraction protocols for isolating fucoidan or FCSPs from different types of brown seaweeds (Tables 1 and 2). The use of different acids—or no acid at all—as well as the differences in extraction time and temperature during the extraction and further purification treatments have generated diverse compositional results and structural suggestions for fucoidan or FCSPs (Tables 1 and 2). The early reports almost unequivocally found that fucoidan mainly contained fucose and sulfate; nevertheless, the chemical composition of the most highly purified, but still crude fucoidan specimen from *Himanthalea lorea* indicated that the fucoidan of this seaweed species contained fucose, galactose, xylose, uronic acids as well as sulfate [8,14]. In the more recent reports, a pretreatment of the seaweed biomass prior to the real extraction treatment has been found to be advantageous to eliminate low molecular components (e.g., phenols); as already mentioned above, an early study used formaldehyde pretreatment [12]. However, more recent reports show that the pretreatment typically involves the use of a mixture of methanol, chloroform and water [22]. Removal of protein has also been considered. This can be facilitated via the use of proteolytic enzymes [28] (or by lead acetate treatment, as used by Percival and Roos in 1950 [8]). Another useful purification procedure has involved transformation of alginate

in the residual biomass into insoluble calcium salts by treatment of the FCSPs specimen with aqueous calcium chloride [19,20].

In conclusion, the use of an array of different extraction and purification techniques appear to have contributed to the confusion that has prevailed about the nature and composition of fucoidan and FCSPs ever since fucoidan was first described by Kylin early in the 20th century [2]. As detailed in the following, we now know that the initial suggestions [29,30] that fucoidan was built of α(1→2) linked L-fucopyranosyl residues were wrong. Fucoidan is built of 1→3-linked α-L-fucopyranosyls or of alternating 1→3- and 1→4-linked α-L-fucopyranosyl residues that may be sulfate substituted. We also know,that "fucoidans" isolated from certain brown algae have completely different structures being composed of sulfated galactofucans with backbones of (1→6)-linked β-D-galacto- and/or (1→2)-β-D-manno-pyranosyl units with (1→3) and/or (1→4)-α-L-fucooligosaccharide branching. The available data thus show that the term "fucoidan" has been used for several different chemical structures and *vice versa* that fucoidan is a term that covers a diverse family of fucose-containing sulfated polysaccharides (Table 1). It is therefore more correct to use the term fucose-containing sulfated polysaccharides (FCSPs) rather than fucoidan as a collective term for these polysaccharides.

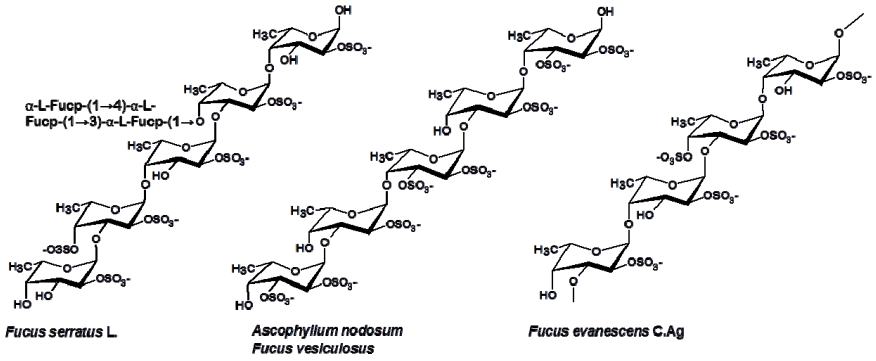
3. Taxonomic Comparison of Fucoidan or FCSPs Structure

3.1. Fucales

In 1993 a revised structure of the polysaccharide backbone of the main FCSP product from *F. vesiculosus* was presented as α(1→3) linked instead of as α(1→2) linked [31]; it was also reported that fucose was attached to the backbone fucan polymer to form branching points, typically one for every 2–3 fucose residues within the chain, still with sulfate groups at position C-4 on the fucose units [31]. However, detailed analysis of the methyl derivatives obtained from partially desulfated *F. vesiculosus* polysaccharides revealed the presence of 2,4-di-*O*-methylfucose as well as 2,3-di-*O*-methylfucose which indicated the presence of both α(1→3) and α(1→4) linked fucose residues [32] (Figure 2). A similar structure was also determined for a FCSPs-derived oligosaccharide of about 8–14 monosaccharide units extracted from *Ascophyllum nodosum* (Fucales) [11] (Figure 2).

More recently, several studies—using highly advanced analytical methods—have documented that fucoidan from brown seaweed in the order of Fucales such as *F. evanescens* and *F. serratus* do indeed contain large proportions of both α(1→3) and α(1→4) glycosidic bonds [20–22] (Figure 2). Structural analysis of a depolymerized low molecular weight fraction of fucoidan from *F. evanescens* by MALDI-TOF and tandem ESI mass spectrometry has moreover shown that this fraction contains oligosaccharides with and without sulfate substitutions and that it mainly consists of α(1→3)-linked fucose residues being esterified with sulfate at C-2 [33]. This more detailed analysis has also revealed the presence of minor components of mixed monosulfated fucooligosaccharides containing both 2-*O*- and 4-*O*-sulfated (1→4) bonded xylose and galactose residues: Xyl-(1→4)-Fuc, Gal-(1→4)-Fuc, Gal-(1→4)-Gal-(1→4)-Fuc, Gal-(1→4)-Gal [24]. Glucuronic acid (GlcA) was also detected as being a part of the non-sulfated fucooligosaccharides: Fuc-(1→3)-GlcA, Fuc-(1→4)-Fuc-(1→3)-GlcA, Fuc-(1→3)-Fuc-(1→3)-GlcA respectively [33].

Figure 2. Typical structure of fucoidan (FCSPs) obtained from some brown seaweed species in the order of Fucales. The L-fucopyranose backbone of the fucoidan (FCSPs) extracted from *A. nodosum* and *F. vesiculosus* is connected by alternating α(1→3) and α(1→4) linkages [11]; The FCSPs from *F. evanescens* have a similar backbone built up with sulfate substituted at the 2- and 4-position of the fucose residues [20] (only sulfate substitutions on C-2 of fucose are shown in the Figure). Acetate substitutions may also be found at the C-4-position of 3-linked fucose and at C3 of 4-linked fucose units [22] (acetate substitutions not shown in the figure). For *F. serratus* L. a possible fucoside side chain at C-4 is also shown.

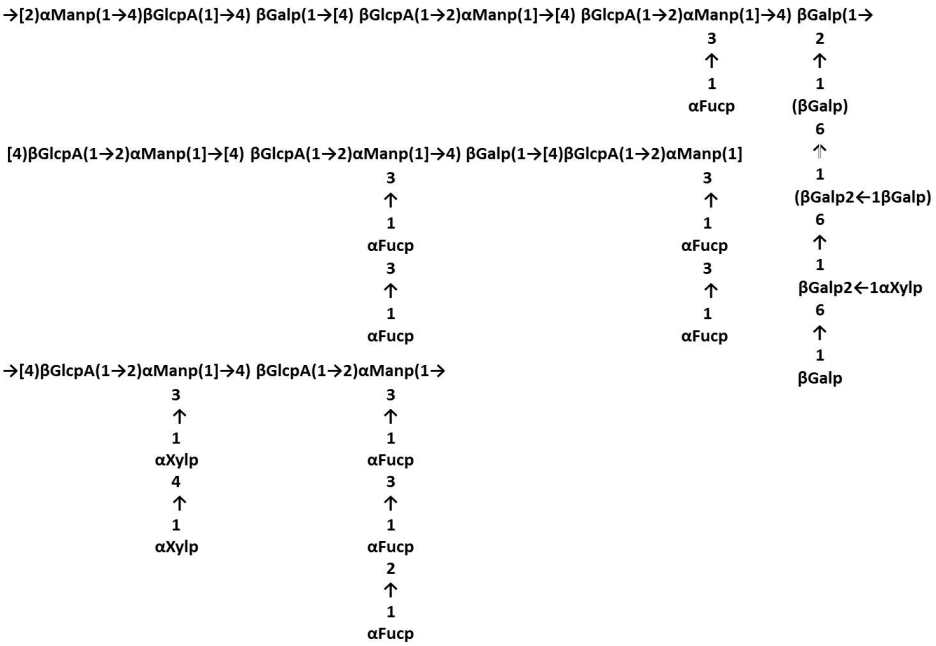


Brown seaweed species in the order of Fucales have also been reported to contain very complex FCSPs structures having fucose and galactose in comparable amounts; these structures are generally referred to as sulfated galactofucans and are predominantly found among *Sargassum* species [18,34,35]. These sulfated galactofucans are mainly built of (1→6)-β-D-galactose and/or (1→2)-β-D-mannose units with branching points formed by (1→3) and/or (1→4)-α-L-fucose, (1→4)-α-D-glucuronic acid, terminal β-D-xylose and sometimes (1→4)-α-D-glucose [18]. Early studies also reported the existence of fucoglucuronans having a backbone of glucuronic acid, mannose and galactose residues with side chains of neutral and partially sulfated residues of galactose, xylose and fucose; notably present in *Sargassum linifolium* [13]. More recently, the FCSPs of this type extracted from the brown seaweed *Sargassum stenophyllum* (Fucales) were grouped into two different types: type I was found to contain a relatively high percentage of α-D-glucuronic acid and relatively few sulfate groups, while type II contained relatively small amounts of α-D-glucuronic acid and a high percentage of sulfate [18]. The type I polysaccharides were composed of a linear backbone formed mainly by (1→6)-β-D-galactose and/or (1→2)-β-D-mannose with branching chains formed by (1→3) and/or (1→4)-α-L-fucose, (1→4)-α-D-glucuronic acid, while in the type II polysaccharides the backbone was mainly built of short galactan chains [18].

Corresponding structures were observed in FCSPs fractions from *Hizikia fusiforme* a.k.a. *Sargassum fusiforme* (Fucales). These structures were separated by ion exchange chromatography after the FCSPs had been obtained via hot aqueous extraction, followed by ethanol and $CaCl_2$ precipitation (Table 2). These chromatographically purified fractions predominantly contained fucose, mannose, galactose, uronic acid and sulfate [23] (Figure 3). In accordance with the findings of Duarte *et al.* [18]

the structural analysis of one of the main fractions purified by ion exchange chromatography indicated that the sulfate groups might be found in any position on the galactose/mannose backbone or on the fucose units (Figure 3). The sulfate groups in the FCSPs in this fraction, which had been isolated after 3 rounds of extraction in hot water (70 °C), then ethanol and $CaCl_2$ precipitation prior to chromatography (Table 2), were mainly found at C-6 of [→2,3)-Man-(1→], at C-4 and C6 of [→2)-Man-(1→] and at C-3 of [→6)-Gal-(1→] [23]. On the fucose, the sulfate groups were substituted at C-2, C-3 or C-4, while some fucose residues had two sulfate groups [23]. The core of these *S. fusiforme* FCSPs was mainly composed of alternating units of [→2)-α-D-Man-(1→] and [→4)-β-D-GlcA-(1→], with a minor portion of [→4)-β-D-Gal-(1→] units, and the branching points were at C-3 of [→2)-Man-(1→], C-2 of [→4)-Gal-(1→] and C-2 of [→6)-Gal-(1→], respectively [23] (Figure 3).

Figure 3. Suggested structures of the FCSPs (fucoidan) from *H. fusiforme* [23] also known as *Sargassum fusiforme* (Fucales); sulfate substitutions not shown. The structures also represent typical FCSPs structures from other *Sargassum* spp. [18].



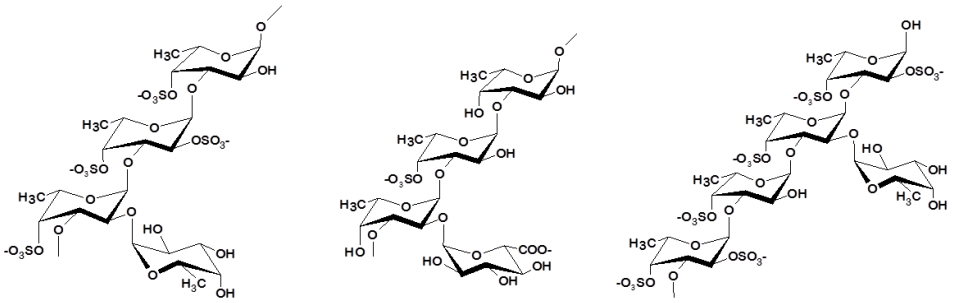
3.2. Laminariales and Other Brown Seaweed

Various structures of FCSPs from brown seaweeds of the order of Laminariales have also been reported [36]. The available data indicate that the FCSPs derived from this seaweed order contain small amounts of other monosaccharides besides fucose. Interestingly, polysaccharides containing significant amounts of fucose and galactose and which seem to be compositionally and structurally similar to the fucoidan from Fucales brown seaweeds appear to be prevalent [26,35]. Structural analysis was conducted on the FCSPs from the sporophyll *Undaria pinnatifida* (Laminariales) and it

was found that the FCSPs had a high fucose/galactose ratio, high uronic acid, and low sulfate content. The most abundant fucopyranosyl units were substituted at the 3-; 2,3-; or 2,3,4-positions whereas fucose residues with substitutions at the 3,4- or 4-positions were less abundant [26]. The galactopyranosyl units were predominantly substituted at the 3- or at both the 3,4-positions [26].

FCSPs isolated from *Chorda filum* (Laminariales) have been shown to consist of a poly-α(1→3)-fucopyranose backbone with a high degree of branching mainly as α(1→2)-linked single α-L-fucopyranosyl residues (Figure 4) [24,25]; the fucopyranosyl residues were found to be sulfated mainly at C-4 and sometimes at the C-2 position, whereas some of the α(1→3)-linked fucose residues were shown by NMR to be C-2 acetylated [25]. A similar structure has been reported by Usov *et al.* [24] for the FCSPs isolated from *L. saccharina* (Laminariales) which are mainly built of (1→3)-linked α-L-fucopyranose with sulfation at C-4 and sometimes at the C-2 position or with possible α-L-fucopyranosyl at C-2 (Figure 4). This FCSPs structure has also been found to be present in the body wall layer of the sea cucumber *Ludwigothurea grisea* (a marine invertebrate). The FCSPs of the sea cucumber body wall are essentially built of an α-(1→3)-fucopyranose backbone [37]. NMR analysis has indicated that 2,4-di-sulfo-L-fucopyranose and unsubstituted fucopyranose are present in equal proportions, and that 2-mono-sulfo-L-fucopyranose is present in twice that proportion [33]. The FCSPs from *Lessonia vadosa* (Laminariales) have also been studied by NMR spectroscopy and the data indicate that the polysaccharides are mainly composed of α(1→3)-bonded fucopyranose residues sulfated mainly at position C-4 and partially at position C-2 [38].

Figure 4. Structural motifs of FCSPs (fucoidan) from some brown seaweed species of the order Laminariales and Chordariales. FCSPs of *Chorda filum* and *Laminaria saccharina* consist of a poly-α-(1→3)-fucopyranoside backbone with sulfate mainly at C-4 and sometimes at the C-2 position; some of the backbone fucose residues may be acetylated at C-2 (not shown) [24,25]. *Cladosiphon okamuranus* derived FCSPs also consist of a backbone of α(1→3)-linked-L-fucopyranose residues with sulfate substitutions at C-4 and/or with α(1→2)-linked single α-L-fucopyranosyl substitutions and vicinal glucuronic acid substitutions. Some of the side chain fucose residues may be O-acetylated (not shown) [14].



Other algal fucoidans whose structures contain the same α(1→3)-backbone of fucose residues have been found in *Analipus japonicas* (Ectocarpales), *Adenocystis utricularis* (Ectocarpales) and

Cladosiphon okamuranus (Chordariales) [14,15,39], but the FCSPs from these brown algae also appear to contain other monosaccharides than fucose (Table 2). The detailed structural elucidation of the FCSPs from *C. okamuranus* (Chordariales) confirmed that this product was made up of a linear backbone of α(1→3)-fucopyranose units with a portion of the fucose residues carrying sulfate substitutions at C-4 but some of the fucose residues have also been found to be O-acetylated (Figure 4). The *C. okamuranus* FCSPs may also contain α-glucuronic acid substitutions at the C-2-positions of those backbone fucose residues that are not substituted by a sulfate group [14] (Figure 4).

Methylation analysis, desulfation and NMR spectroscopy of the FCSPs fractions from *Adenocystis utricularis* (Ectocarpales) showed that these FCSPs contained the same α-(1→3)-fucopyranose backbone as that found in *Chorda filum* and *Laminaria saccharina* FCSPs, and that the fucopyranosyl units were mostly sulfated at C-4, and branched at C-2 with non-sulfated fucopyranosyl units; the galactan moiety, which was also present, was predominantly found to be a backbone structure of (1→3) and (1→6) D-galactopyranose units with sulfation mostly on C-4 [15]. Later, a similar structure was found in FCSPs extracted from *Analipus japonicas* (Ectocarpales) [39].

The relatively large variations in the reported compositional and structural properties of the FCPSs extracted from different brown seaweed species thus clearly confirm the natural biodiversity of FCSPs notably as exemplified by the structures found in Fucales, e.g., in the *Fucus* sp. and *Sargassum* sp. (Figures 2 and 3). The (1→3)-linked α-L-fucopyranosyl backbone structure, with various extents of sulfate substitutions, is prevalent as the core backbone structure in the majority of the currently analyzed FCSPs. Nevertheless, the reported structural data for FCSPs from different brown seaweed species clearly indicate that there is no consistent basic structure of "fucoidan". It also seems clear that FCSPs extracted from seaweeds under the same order have different composition, and in turn that the structural traits of FCSPs cannot be categorized or predicted according to algal order (Tables 1 and 2).

When assessing the available compositional data the large variation in the composition of the FCSPs products obtained from different extraction methods is evident (Table 2). Recently, we optimized the extraction yields of FCSPs from *Sargassum* sp. by developing a single-step extraction procedure. While doing so, we also systematically examined the effects of different extraction parameters (*i.e.*, acid concentration, time, and temperature) on the yields and composition of the FCSPs products obtained [19] (Table 2). All extraction factors affected the FCSPs yield. Lower total FCSPs yields, but higher fucose contents in the products were obtained with shorter extraction time [19]. The work also revealed that classical extraction treatment with HCl at elevated temperature and during extended time, *i.e.*, a procedure similar to the one used by Black *et al.* [9], had a detrimental effect on the FCSPs yield as this treatment apparently disrupted the structural integrity of the fucose-containing polymer and caused degradation of the chains built of fucose residues [19]. Hence, some of the classic methods, employing relatively harsh acid treatments, may in fact have affected the composition and structure of the target FCSP products to different extents, and may have contributed to the prevailing "conflicting views on the nature of fucoidin" recognized already in 1950 by Percival and Ross [8]. A consensus to employ defined extraction protocols for extraction of FCSPs, or at least an agreement among scientists in the field to include a benchmark extraction procedure in their studies, would help to advance the understanding of these intriguing FCSPs substances.

4. Bioactivity of Fucoidan or FCSPs

In recent years, fucoidan or FCSPs from seaweed biomass have been the subject of many scientific studies aiming at assessing their potential biological activities including antitumor and immunomodulatory [40–42], antivirus [43], antithrombotic and anticoagulant [44], anti-inflammatory [45], and antioxidant effects [46], as well as their effects against various renal [47], hepatic [48] and uropathic disorders [49].

Recently, low molecular weight FCSPs have been shown to have therapeutic potential in preventing intimal hyperplasia in both *in vivo* and *in vitro* studies: Contact with low molecular weight FCSPs ("fucoidan") thus increased the migration of human vascular endothelial cells and induced decreased migration of vascular smooth muscle cells *in vitro* [50]. In an *in vivo* rat experiment FCSPs reduced the intimal hyperplasia in the rat aortic wall after balloon injury [50]. Furthermore, an *in vivo* efficacy study of fucoidan films conducted using a rat model showed that during cecal-sidewall surgery a fucoidan film wound healing treatment reduced the adhesion scores by approximately 90% and resulted in 50% to 100% of animals being adhesion free [51].

In this review, the most significant bioactivities of FCSPs, including antitumor and immunomodulatory, anticoagulant and antithrombotic effects will be presented with special focus on the relationship between the FCSP structural features and biological activity.

4.1. Antitumor and Immune-Response Activities

Several different therapeutic strategies such as chemotherapy, radiation therapy, surgery or combinations hereof have been used to treat different types of cancer. Unfortunately, several of these treatments provide only minimal benefits; moreover, there are undesirable complications and long term side effects of the treatments [52,53]. Consequently the quest for potential preventive or therapeutic measures against cancer has been going on for years and recently the focus has been directed towards bioactive compounds of natural origin, including FCSPs from brown seaweeds [1]. Many reports have been published which indicate the antitumor and immune-response modulating activity of FCSPs in both *in vivo* and *in vitro* studies [40–42,54,55].

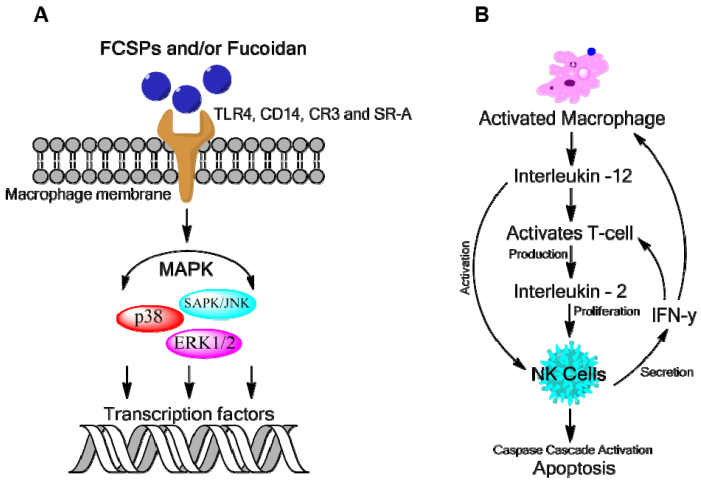
Sulfated polysaccharide fractions from *Sargassum fulvellum*, *S. kjellmanianum*, *L. angustata*, *L. angustata* var. *longissima*, *L. japonica*, *Ecklonia cava*, and *Eisenia bicyclis* have been evaluated for their bioactivities, and they have been found to exert remarkable growth inhibitory activities on Sarcoma-180 cells implanted into mice and to possess antitumor activity against L-1210 leukemia in mice [56–58]. Recently, we reported the potent *in vitro* bioactivity of FCSPs extracted from *Sargassum* sp. and *F. vesiculosus* against lung and skin cancer cell growth [42]. The antitumor mechanism of FCSPs from sporophylls of *Undaria pinnatifida* has been described by Maruyama *et al.* [41,59]. The available findings indicate that antitumor activity of FCSPs may be associated with a significant enhancement of the cytolytic activity of natural killer (NK) cells augmented by increased production of macrophage-mediated immune response signaling molecules [59–61], namely interleukins (IL)-2, IFN-γ and IL-12 [42,59], and induction of apoptosis [42].

Macrophage activation by polysaccharides is mediated through specific membrane receptors. The major receptors reported for polysaccharides recognition in macrophages are glycoproteins including

Toll-like receptor-4 (TLR-4), cluster of differentiation 14 (CD14), competent receptor-3 (CR-3) and scavenging receptor (SR) [61]. Activation of these receptors is mediated by intracellular signaling pathways and the family of mitogen-activated protein kinases (MAPKs) plays a critical role notably in the production of nitric oxide (NO) which can lyse tumors [61]. MAPK family members such as p38 MAPK, extracellular regulated kinase (EKR1/2) and stress-activated protein kinase/c-Jun-*N*-terminal kinase play an important role in the activation of macrophages by polysaccharides such as FCSPs [61,62] (Figure 5A). Activated MAPKs lead to activation of transcription factors resulting in induction of various genes [61]. Activation of macrophages induces the production of cytokines such as interleukin-12 (IL-12) which in turn stimulate the development of T-cells (Figure 5B). T-cells produce interleukin-2 (IL-2) that in turn activates NK cells proliferation. The NK cells themselves produce immunologically important cytokines, notably IFN-γ, which can further provoke the participation of macrophages in the stimulation of T-cell via induction of IL-12 [41,59] (Figure 5B).

NK cells appear to represent a first line of defense against the metastatic spread of blood-borne tumor cells, and normal NK activity may be important in immune surveillance against tumors [63]. NK-mediated killing of target cells by apoptosis is facilitated by activation of caspase cascades (Figure 5B). In tumor bearing mice, FCSPs appear to act as an immunopotentiator leading to increased antitumor effectiveness as exhibited by increased immune response against A20 leukemia cells and a lowering of the tumor size in transgenic (DO-11-10-Tg) mice [41]. Moreover, recent investigations of the immunomodulatory activity of FCSPs in rats with aspirin-induced gastric mucosal damage suggest that the gastro-protective effect of fucoidan against aspirin-induced ulceration may take place through the prevention of elevation of pro-inflammatory cytokines, IL-6 and IL-12 [64].

Figure 5. Proposed mechanism responsible for fucoidan bioactivity: (**A**) Macrophage activation by FCSPs as mediated through specific membrane receptor activation namely TLR-4, CD14, CR-3 and SR which in turn induce intracellular signaling via mitogen-activated protein kinases (MAPKs); (**B**) Activation of macrophages lead to production of cytokines such as IL-12, IL-2 and IFN-γ which enhance NK cell activation that may stimulate T-cell activation further via IFN-γ.



Apoptosis is one of the most prevalent pathways through which FCSPs can inhibit the overall growth of cancer. Previous studies have shown that different types of FCSPs can induce apoptosis in melanoma cells [42], HT-29 colon cancer cells [65], MCF-7 human breast cancer cells [66], and HS-Sultan human lymphoma cells [62]. In human HS-Sultan cells, the apoptosis may occur via activation of caspase-3 [62]; and in MCF-7 cells via caspase-8 dependent pathways [66]. Alternatively, FCSPs induced apoptosis may take place through activation of caspases via both death receptor-mediated and mitochondria-mediated apoptotic pathways [65].

4.2. Anticoagulant and Antithrombotic Activities

The earliest published report describing the anticoagulant activity of fucoidan was published in 1957 [67]. In that report it was shown that a certain fraction of fucoidan from *F. vesiculosus* possessed powerful anticoagulant activity that qualified fucoidan to belong to the group of heparinoids [67]. Heparin is a biomolecule containing highly sulfated glucosaminoglycan that is widely used as an injectable anticoagulant. It has been reported that the anticoagulant mechanisms of fucoidan are related to both antithrombin and heparin cofactor II-mediated activity [68,69], but the mechanisms by which fucoidan exerts anticoagulant activity remain controversial [32]. Hence, any possible relations between the physical and chemical properties, the structure, and the anticoagulant activity of fucoidan remain to be firmly established. The uncertainties are mainly due to the structural variation of fucoidan between algal species, but most likely also a result of the different extraction methodologies employed to isolate FCSPs that appear to have produced FCSPs of different composition, structure, and size, which have given rise to conflicting results in the detailed studies of mechanisms of anticoagulant activity [32,70].

Results obtained using the so called activated partial thromboplastin time assay (APTT) have strongly indicated that FCSPs from *F. vesiculosus* have specific anticoagulant activity. Comparable results have been obtained in two independent studies using FCSPs dosages equivalent to 9–13 U/mg *versus* 167 U/mg for heparin; and 16 U/mg *versus* 193 U/mg heparin, respectively [71,72]. When FCSPs samples isolated from nine brown seaweed species were tested for anticoagulant activities, the APTT results were significant at 12–38 U/mg as compared to at 167 U/mg for heparin [73]. A remarkable finding for anticoagulant action was also reported by Kitamura *et al.* [74], who showed that a FCSPs fraction from *L. angustata* var. *longissima* (Laminariales) had antithrombin activity at 200 U/mg, equivalent to a dose of 140 U/mg heparin. The particular FCSPs fraction having anticoagulant activity had a molecular weight of ~21–23 kDa and contained fucose-galactose-sulfate at a ratio of 9:1:9 with the sulfate substitutions at C-4 of the fucose residues [74].

It has been postulated that it is not a specific structural trait that determines fucoidan's ability to elicit anticoagulant activity, but rather that the anticoagulant effect is due to a multitude of structural features including monosaccharide composition, molecular weight, sulfation level, and position of sulfate groups on the main chain of the polysaccharide [69,75–77]. The comprehensive study of the anticoagulant activity of fucoidan by Cumashi *et al.* [21] also noted that neither the content of fucose and sulfate nor other structural features affected the anticoagulant efficacy. FCSPs from *C. okamuranus* (Chordariales) have been reported to exert virtually no anticoagulant effect, and this could be due to the low amount of sulfate in its polymer backbone and/or the presence of vicinal branching points formed by 2-*O*-α-D-glucuronyl substituents (Figure 4) [21,14]. On the other hand, the concentrations

of C-2 sulfate and C-2,3-disulfated sugar residues (Figure 2) have been reported to be a common structural feature for fucoidan anticoagulant activity [11]. The anticoagulant activity of high molecular weight FCSPs from *Ecklonia kurome* were thus reported to be dependent on both molecular weight and sulfate content [76]: Higher molecular weight FCSPs (*i.e.*, 27 and 58 kDa) showed higher anticoagulant activity than lower molecular weight FCSPs (*i.e.*, ≤10 kDa); and FCSPs samples having a high molar ratio of sulfate to total sugar residues were found to exhibit inhibitory effects on fibrinogen clotting by thrombin reaction [76]. These results were supported by data reported by Haroun-Bouhedja *et al.* [78] who reported a relationship between the extent of sulfate group substitutions and the biological activities of fucoidan. The anticoagulant activity of low molecular weight (LMW) fucoidan, *i.e.*, MW < 18 kDa was thus found to decrease with decreasing degree of sulfation, and very low-sulfate (<20%) or desulfated LMW fucoidan lost its anticoagulant activity, but retained some antiproliferative activity on CCL39 fibroblast cells [78]. In contrast, LMW fucoidan with sulfate content higher than 20% was found to exert profound anticoagulant activity as well as antiproliferative effects on fibroblast cell line CCL39 cells in a dose-dependent fashion [78].

Some studies suggest that also the sugar composition (e.g., fucose, galactose, mannose, etc.) or the type of oligo- or polysaccharides of the FCSPs may play an important role for anticoagulant activity [75,79]. However, the series of investigations conducted by Pereira *et al.* [32,80,81] indicated that a 2-sulfated, 3-linked α-L-galactan, but not α-L-fucan, was the potent thrombin inhibitor mediated by anti-thrombin of heparin cofactor II. These findings have however also pointed out that it is not necessarily the sugar composition but rather the sulfate substitutions on the sugars that determine the anticoagulant activity of fucoidan—or both [82].

Most of the reported studies were carried out with crude, diverse and complex FCSPs obtained via extraction from brown seaweeds as opposed to being chemically well defined structures. For this reason it is not easy to identify a structure *versus* activity relationship because of the presence of highly branched portions and the complex distributions of sulfate and acetyl groups in algal FCSPs. This aspect was attempted resolved by use of invertebrate polysaccharides [83]. The data obtained indicated that regular, linear sulfated α-L-fucans and sulfated α-L-galactans express anticoagulant activity, which is not simply a function of charge density, but critically dependent on the pattern of sulfation as well as monosaccharide composition. Sulfated α-L-fucans and fucosylated chondroitin sulfate were also shown to elicit antithrombotic activity when tested on *in vivo* models of venous and arterial thrombosis in experimental animals [83].

4.3. Bioactivities and Oversulfation of FCSPs

In 1984 crude FCSPs fractions from *Sargassum kjellmanianum* were prepared in order to investigate the influence of the sulfation levels on the survival of L-1210 leukemia bearing mice; and on the growth of Sarcoma-180 cells [58]. The study showed that the fraction with the highest sulfation was the most effective against L-1210 leukemia bearing mice and it produced an increase in life span of 26%. On the other hand, this particular FCSPs fraction was also less effective in inhibiting growth of Sarcoma-180 cells subcutaneously implanted into mice [58].

Since then, several investigations have focused on the effect of oversulfation of FCSPs on biological activity [54,78,84–89]. Oversulfated FCSPs may be obtained by further sulfation of native FCSP

molecules using dimethylformamide as solvent and a sulfur trioxide-trimethylamine complex as the sulfating agent [86]. The inhibitory effects of such oversulfated FCSPs were investigated on the invasion of Murine Lewis Lung Carcinoma cells through a reconstituted membrane basement fragment, socalled laminin [86]. Oversulfated FCSPs were found to be the most potent inhibitor of tumor cell invasion and were also, in particular, found to inhibit tumor cell adhesion to laminin better than native and desulfated FCSPs. The most potent oversulfated FCSP structures had sulfate groups on both the C-3 and C-4 positions of the fucose units; hence, the particular spatial orientation of the negative charges in the FCSPs molecules may also be an important determinant of bioactivity [86]. The study did not allow firm conclusions to be drawn with respect to mechanisms of action, but it was suggested that the increased negative charge resulting from oversulfation might promote the formation of FCSPs-protein complexes involved in cell proliferation, in turn suppressing cell growth [86].

When the importance of the spatial orientation of the negative charges on the FCSPs was investigated in more depth it was confirmed that this feature plays a major role in determining the binding potency of FCSPs to vascular endothelial growth factor 165 ($VEGF_{165}$) [88]. Both native and oversulfated FCSPs have been tested for their anti-angiogenic actions *in vivo* and for their *in vitro* anti-proliferative effects against B16 melanoma cells, Sarcoma-180 and Lewis lung carcinoma cells: The interaction of oversulfated FCSPs with $VEGF_{165}$ occurred with high affinity and resulted in the formation of highly stable complexes, thereby interfering with the binding of $VEGF_{165}$ to vascular endothelial growth factor receptor-2 (VEGFR-2). The results showed that both native and oversulfated FCSPs were able to suppress neovascularization in mice implanted Sarcoma-180 cells; and that both FCSPs types inhibited tumor growth through the prevention of tumor-induced angiogenesis, but the data indicated that sulfation tended to give more potent effects [88].

Native and oversulfated FCSPs derived from *Cladosiphon okamuranus* (Chordariales) were analyzed using ^{1}H NMR spectroscopy and it was suggested that whereas natural sulfation produced 4-mono-*O*-sulfo-L-fucopyranose the oversulfated FCSPs contained 2,4-di-, 2-mono-, and 4-mono-*O*-sulfo-L-fucopyranose [89]. It was also suggested that sulfate content and the positioning of sulfate groups, e.g., 2,4-di- *vs.* 4-mono, might be important for the anti-proliferative activity of fucoidan in a human leukemia cell line (U937), an effect which is presumed to take place via induction of apoptosis associated with activation of caspase-3 and -7 [89].

The effects of oversulfation of low and high molecular weight FCSPs derivatives from *F. vesiculosus* and heparin on lipopolysaccharide (LPS)-induced release of plasminogen activator inhibitor-1 (PAI-1) from cultured human umbilical vein endothelial cells (HUVEC) were examined by Soeda *et al.* [87]. Their study demonstrated that all oversulfated FCSPs derivatives including high molecular weight derivatives of 100–130 kDa were effective in suppressing the LPS-induced PAI-1 antigen, and supported an important role of the degree of sulfation for bioactivity [87].

The correlation of oversulfation and conformation of molecular sizes of FCSPs for anticancer activity using human stomach cancer cell lines AGS was evaluated recently for FCSPs isolated from dried Undaria pinnatifada FCSPs [54]. The data showed that the oversulfated, low molecular weight FCSP derivatives increased the inhibition of cell growth, while the growth inhibition was less for native, high molecular weight FCSPs and for oversulfated high molecular weight FCSPs [54]. The differences were suggested to be a result of the smaller molecular weight fractions having a less

compact conformation than the higher, which may have allowed a higher extent of sulfate substitution to occur during oversulfation.

5. Conclusions

Fucoidan—or FCSPs—are an important group of polysaccharides that show remarkable biological actions notably anticoagulant, antitumor and immune-response activities. Despite intensive research, the exact correlation between the bioactivity and the structural molecular features of FCSPs—which vary depending on seaweed species and extraction methodology—has yet to be clarified.

The preservation of the structural integrity of the FCSPs molecules nevertheless appears crucial for maintaining the biological properties and it has been clearly shown that the extraction treatment employed affects the composition and thus the structural features of the FCSPs substances.

The diverse structures and varied chemical composition of FCSPs may have hindered the development of an in-depth understanding of the precise properties of significance for specific bioactivity effects.

Important structural issues for bioactivity appear to include the degree of sulfation and the size of the FCSP molecules. Oversulfated FCSPs have thus been found to be potent inhibitors of tumor cell invasion compared to desulfated native FCSPs. Low molecular weight FCSPs have been shown to be effective in inhibiting human stomach cancer cell growth and to exert anticoagulant activity provided that the extent of the degree of sulfation was relatively high. Loss of anticoagulant activity has been observed with decreasing degree of sulfation, although anti-proliferative effects on fibroblast cell lines were retained.

Undoubtedly, the presence of impurities influences the biological properties of FCSPs and therefore may currently hinder our full understanding of the biological activity of fucoidan or FCSPs. Hence the development of standard extraction procedures for FCSPs including hydrolysis treatment, purification and fractionation methodology, preferably with specific steps adapted to the particular botanical order of the seaweed, will generate a better, common basis for analysis and understanding of bioactivities and the mechanisms determining the bioactivities of FCSPs. On this basis it may even be possible to target specific structural features and in turn tune the extraction procedure to obtain specific bioactivities via the use of targeted extraction methodologies.

Despite the availability of early, seminal studies of the extraction of FCSPs from brown seaweeds the understanding of the complex structures of FCSPs, is far from complete.

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3 Extraction of FCSPs

Brown seaweeds in the class of Phaeophyceae are excellent sources of sulfated polysaccharides notably FCSPs (Shanmugam and Mody, 2000; Abdel-fattah et al., 1974). FCSPs comprise families of polydisperse heterogeneous molecules based on L-fucose, D-xylose, D-glucuronic acid, D-mannose, and D-galactose. Fucoidan is part of a group of FCSPs that consists almost entirely of fucose and ester sulfate (Percival and McDowell, 1967), have a backbone of (1→3)-linked α-L-fucopyranosyl or of alternating (1→3)- and (1→4)-linked α-L-fucopyranosyl residues. Nevertheless, we now know that sulfated galactofucans with backbones of (1→6)-β-D-galacto- or (1→2)-β-D-mannopyranosyl units with fucose or fuco-oligosaccharide branching and/or glucuronic acid, xylose, or glucose substitutions that could be considered fucoidans (Nishino et al., 1994). It has long been known that cell wall polymers of brown seaweed are complex; hence, extraction poses a challenge because the yield and chemical nature of the polysaccharides recovered from such seaweeds are markedly influenced by the conditions used to extract them.

Early reports show that treatment with dilute acid at ambient or slightly elevated temperature has been a preferred first step in extraction protocols for isolating fucoidan or FCSPs from different types of brown seaweed. The use of different acids or no acid at all as well as the differences in extraction time and temperature during extraction and further purification treatments have generated varying compositional results and structural suggestions for fucoidan and FCSPs. The early reports almost unequivocally found that fucoidan mainly contained fucose and sulfate; nevertheless, the chemical composition of the most highly purified but still crude fucoidan specimen from *H. lorea* indicated that the fucoidan of this seaweed species contained fucose, galactose, xylose, uronic acid, and sulfate (Percival and Ross, 1950; Percival, 1968).

3.1 Extraction methods

Extraction of brown seaweed fucans generally involves multiple extended aqueous extractions, usually with hot acid, and may include calcium addition to promote alginate precipitation (Chizhov et al., 1999; Marais et al., 2001). It has long been known that extraction time, temperature, and acid concentration/pH may influence both the yield and the composition of the resulting fucans (Li et al., 2008; Black et al., 1952). Hence, Black et al. (1952) reported how the use of different extraction methods influenced the fucose quantity with a disparity of 20–80% of the total fucose of *F. vesiculosus*. The influence of extraction method on fucan yield is further exemplified by

fucoidan yield data from *Laminaria japonica*: the yield was only 1.5% of the DW of the seaweed when extracted using alkaline solution at 95°C for 2 h (Sakai et al., 2002) but was 2.3% DW when the extraction was done using water in an autoclave at 120°C for 3 h (Wang et al., 2008). However, the maximal yield of brown seaweed fucans is typically in the range of 5–7% DW. Fucoidan yields from *F. vesiculosus* have thus been reported to be 7.0% DW, while the yields obtained from *Sargassum horneri* and *Undaria pinnatifida* were found to be 5.2% and 6.8% DW, respectively (Kuda et al., 2002).

3.2 Single-step extraction of FCSPs

This section is an extended elucidation of Paper 1 and concerns designed optimization of a single-step extraction of FCSPs from *Sargassum* sp.

3.2.1 Relevance

Brown seaweed fucans have been reported to possess bioactive properties (Chevolot et al., 1999). Acquiring this valuable compound from non-commercially important seaweed material is of the outmost interest. *Sargassum* sp. is an unexploited brown seaweed, belonging to class Phaeophyceae, which grows wildly almost worldwide in abundance; hence, it is considered a nuisance seaweed. To initially assess the possible use of *Sargassum* sp. as an FCSP source, we wanted to systematically evaluate the influence of the extraction parameters (i.e., acid concentration, time, temperature) and maximize the yield while attempting a relatively mild treatment.

3.2.2 Hypotheses and objectives

The effect of different extraction treatment conditions on FCSP yield and composition was evident, especially in early seminal studies about fucoidan extraction (Black et al., 1952), which recommended the use of a 3-step hot acid extraction procedure. Furthermore, 4× $CaCl_2$ treatment for 5 h at 85°C for the extraction of fucoidan was found to be remarkably useful (Bilan et al., 2002). Nevertheless, repetitive extractions of FCSPs for several hours are probably not necessary since it could generate variations on the chemical nature of polysaccharides, thus influencing its yield and structural integrity.

On the other hand, single-step extraction of FCSPs using optimized parameters (i.e., acid concentration, time, temperature) is sufficient to obtain considerable yield while conserving polysaccharide integrity. Nonetheless, there is limited systematic information about the influences and apparently complex interactions of extraction parameters on fucose-containing sulfated

polysaccharide yield. It is our proposition that more focus should be directed to the extraction and purification processes to obtain consistent protocols that account for the biodiversity of FCSPs from different seaweeds and retain the structurally significant features of the specific bioactivity properties of FCSPs. The development and use of such consistent extraction procedures would also help achieve a better understanding of the structure-function relationships of FCSPs.

Therefore, the objectives of this study were to optimize the extraction of FCSPs while systematically examining the effects of treatments using different acids, reaction times, and temperature on its yield from a *Sargassum* sp. seaweed. We compared the composition of the extracted polysaccharides obtained using the final yield-optimized one-step extraction procedure as well as an analogous two-step extraction procedure to those obtained with the classical state-of-the art multi-step fucoidan extraction methods of Black et al. (1952) and Bilan et al. (2002).

3.2.3 Result highlights

FCSP extraction parameters significantly affect yield: acid concentration has negative effects, while both time and temperature exert positive effects. The optimized single-step extraction was achieved by the statistically designed optimal extraction procedure of 0.03 M HCl, 90°C, and 4 h with a maximal fucoidan yield of approximately 7% DW. The influenced of extended extraction time was evident on polysaccharide composition: the fucose and sulfate content of fucoidan decreased as extraction time increased, while glucuronic acid content increased. The results indicated that obtaining a high-yield FCSP with relatively high fucose content in a single-step extraction was a compromise, and the data confirmed that a shorter extraction time was required to obtain a high fucose yield, while 3-h extraction time was the best compromise to achieve high polysaccharides yield and high fucose levels of FCSP from *Sargassum* sp. seaweed.

The data from this study demonstrated the vulnerability of FCSPs to harsh extraction conditions and confirmed that the extraction methods significantly influence both the yields and the chemical nature of polysaccharides recovered from extraction.

3.2.4 Consideration and justification

Several brown seaweed species could be used as source materials for the production of FCSPs. Nevertheless, *Sargassum* sp. was chosen in this study for the following reasons: it is a highly invasive seaweed species that is abundant worldwide; no commercial application is yet firmly established, thus its commercial value is very low; and the use of *Sargassum* sp. for the production of bioactive compounds such as fucoidan will add value to it.

The main message of the work was that the yields and chemical nature of the polysaccharides were clearly affected by extraction treatment. Apart from highlighting the effects of the extraction parameters and their interactions, this work provides a novel approach to defining milder extraction conditions and reveals that the current state-of-the art extraction methods that involve multiple steps and harsh acid and temperature conditions partly degrade the FCSPs.

A sulfated fucan (a fucoidan) may occur in *Sargassum* sp., but no evidence in this present study to prove that L-fucose comprised the backbone of a significant component of the preparation; as such, the term fucoidan may not reasonably be applied to the isolated product. One could interpret the evidence as indicating that the product isolated contained a sulfated fucose-containing heteroglycan and possibly had a glucuronan primary structure with extensive fucosyl side branches that are cleaved and lost as extraction time increases. In the absence of more direct indication for a fucan structure, it is preferable to refer to the extracted product as fucose-containing sulfated polysaccharides (FCSPs).

Based on the simple and practical method for recovering a suite of complex sulfated polysaccharides from *Sargassum* sp. established in this work, we can, therefore, conclude that product yield and chemical composition are strongly affected by extraction method. An optimized one-step extraction treatment to obtain high yields of FCSP from *Sargassum* sp. was developed, and the effect of different treatment parameters on polysaccharide integrity was established. The evidence presented in this study (Paper 2) shows that the extracted polysaccharide product is heterogeneous at any time it is analyzed, although the composition varies with extraction duration. The monomeric composition shows that fucose and sulfate were important components of the polysaccharide mixture as isolated (Paper 2). The results confirmed that *Sargassum* sp. may be a good source of FCSPs. The data also demonstrated the vulnerability of FCSPs to harsh extraction conditions and confirmed that extraction method significantly influences FCSP composition and yield. We strongly emphasize this point as it has a major bearing on any study in which such products are being evaluated for biological activity. It is our belief that the model obtained may be applied to other fucoidan-containing brown seaweeds.

3.3 Paper 2: Designed optimization of single-step extraction of FCSPs from *Sargassum* sp.

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Designed optimization of a single-step extraction of fucose-containing sulfated polysaccharides from *Sargassum* sp.

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Abstract Fucose-containing sulfated polysaccharides can be extracted from the brown seaweed, *Sargassum* sp. It has been reported that fucose-rich sulfated polysaccharides from brown seaweeds exert different beneficial biological activities including anti-inflammatory, anticoagulant, and anti-viral effects. Classical extraction of fucose-containing sulfated polysaccharides from brown seaweed species typically involves extended, multiple-step, hot acid, or $CaCl_2$ treatments, each step lasting several hours. In this work, we systematically examined the influence of acid concentration (HCl), time, and temperature on the yield of fucose-containing sulfated polysaccharides (FCSPs) in statistically designed two-step and single-step multifactorial extraction experiments. All extraction factors had significant effects on the fucose-containing sulfated polysaccharides yield, with the temperature and time exerting positive effects, and the acid concentration having a negative effect. The model defined an optimized single-step FCSPs extraction procedure for *Sargassum* sp. (a brown seaweed). A maximal fucose-containing sulfated polysaccharides yield of ~7% of the *Sargassum* sp. dry matter was achieved by the optimal extraction procedure of: 0.03 M HCl, 90°C, 4 h. HPAEC-PAD analysis confirmed that fucose, galactose, and glucuronic acid were the major constituents of the polysaccharides obtained by the optimized method. Lower polysaccharide yield, but relatively higher fucose content was obtained with shorter extraction time. The data also revealed that classical multi-step extraction with acid ≥0.2 M HCl at elevated temperature and extended time had a detrimental effect on the FCSPs yield as this treatment apparently disrupted the structural integrity of the polymer and evidently caused degradation of the carbohydrate chains built up of fucose residues.

Keywords Fucoidan · *Sargassum* · Brown seaweed · Fucose · Bioactive compound · Extraction method

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Introduction

Fucose-containing sulfated polysaccharides, or "fucoidan", from brown algae may contain differing glycosidic linkages and are variously substituted with acetate and side branches containing fucose or other glycosyl units. These fucose-containing sulfated polysaccharides (FCSPs) can be extracted from brown seaweed species such as *Fucus*, *Laminaria*, and *Sargassum* (Li et al. 2008; Mori and Nisizawa 1982). A range of biological activities have been attributed to FCSPs including anti-tumoral (Zhuang et al. 1995), anti-viral (Adhikari et al. 2006; Trinchero et al. 2009), anti-inflammatory (Blondin et al. 1994); and notably anticoagulant effects (Nardella et al. 1996). The potential pharmaceutical and medical applications of FCSPs have recently directed special interest into utilization of brown seaweeds as a source of FCSPs (Li et al. 2008; Cumashi et al. 2007).

Some brown seaweed FCSPs have a backbone of 3-linked α-L-fucopyranose, while others have a backbone of alternating 3- and 4-linked α-L-fucopyranose residues and sulfated galactofucans (Bilan and Usov 2008). The sulfated galactofucans are prominently found in various *Sargassum* species (Duarte et al. 2001; Zhu et al. 2003). These sulfated galactofucans are mainly built of (1→6)-β-D-galactose and/or (1→2)-β-D-mannose units with branching points

formed by (1→3) and/or (1→4)-α-L-fucose, (1→4)-α-D-glucuronic acid, terminal β-D-xylose and sometimes (1→4)-α-D-glucose (Duarte et al. 2001). Early studies also reported the existence of fucoglucuronans having a backbone of glucuronic acid, mannose, and galactose residues with side chains of neutral and partially sulfated residues of galactose, xylose, and fucose; these supposed fucoglucuronans have been reported to be present in *Sargassum linifolium* (Abdel-Fattah et al. 1974).

Extraction of fucose-containing sulfated polysaccharides from brown seaweeds generally involves multiple, extended aqueous extractions, usually with hot acid (hydrochloric acid), and may include calcium addition to promote alginate precipitation (Chizhov et al. 1999; Marais and Joseleau 2001). It has long been known that extraction time, temperature, and acid concentration/pH may influence both yields and composition of the resulting fucoidan or FCSPs (Li et al. 2008; Black et al. 1952). Already in 1952, Black et al. (1952) reported how the use of different extraction methods influenced the quantity of fucose with a disparity of 20% to 80% of total fucose of *Fucus vesiculosus* fucoidan and 20% to 55% of total fucose of *Pelvetia canaliculata* fucoidan (Black et al. 1952). The influence of extraction method on fucose-containing sulfated polysaccharides yield is further exemplified by data for the yield from *Laminaria japonica*: the yield was only 1.5% of the dry weight (DW) of the seaweed when extracted with alkaline solution at 95°C for 2 h (Sakai et al. 2002), but 2.3% DW when the extraction was done with water in an autoclave at 120°C for 3 h (Wang et al. 2008). On the other hand, the FCSPs notably fucoidan yield of a combined extract of *F. evanescens* was 12.9% DW when extracted four times with 2% $CaCl_2$ solution at 85°C for 5 h (Bilan et al. 2002), while cold extraction with 0.4% HCl at 25°C for 5 h yielded 12.0% DW (Zvyagintseva et al. 1999). Typically, the maximum FCSPs yields from (dried) brown seaweeds range from 5–7% DW. Fucoidan yields extracted from *F. vesiculosus* have thus been reported to be 7.0% DW; while the fucoidan yields obtained from *Sargassum horneri* and *Undaria pinnatifida* were found to be 5.2% DW and 6.8% DW, respectively (Kuda et al. 2002). Despite the existence of early seminal studies about FCSPs extraction, notably Black et al. (1952), that recommended the use of a three-step hot acid extraction procedure, there is only limited systematic information about the influences and apparently complex interactions of extraction parameters acid, temperature and time on fucose-containing sulfated polysaccharides yield.

Sargassum is an unexploited brown seaweed genus in the Phaeophyceae which grows wildly in enormous quantities almost all over the world, but it is particularly abundant along the coastal regions in south East Asia, where members of this genus are considered as nuisance seaweeds. In order to initially assess the possible use of *Sargassum* sp. as a source of FCSPs, we wanted to evaluate systematically the influence of the extraction parameters, i.e., acid concentration, time, temperature, and maximize the FCSPs yields, while at the same time attempt a relatively mild extraction procedure. In this present work, we therefore systematically examined the effects of different combinations of acid, reaction time and temperature on the fucose-containing sulfated polysaccharides yields from a *Sargassum* sp. obtained from Vietnam. We also compared the composition of the extracted polysaccharides obtained by the final yield-optimized one-step extraction procedure as well as an analogous two-step extraction procedure to those obtained with the classical, state-of-the art multi-step fucoidan extraction methods of Black et al. (1952) and Bilan et al. (2002).

Materials and methods

Chemicals Hydrochloric acid 37%, D-glucose and D-xylose were purchased from Merck. Ethanol 99.8%, trifluoracetic acid 99% (TFA), trichloroacetic acid 99%, diethyl ether, $CaCl_2$, Na_2SO_4, $BaCl_2$, L-arabinose, L-rhamnose, D-galactose, L-fucose, D-mannose and D-glucuronic acid were from Sigma-Aldrich (Germany). Agarose D-2 was obtained from Hispanagar (Spain). All chemicals used were analytical grade. Dried *Sargassum* sp. was obtained from the company Viet Delta Co. Ltd. (Ho Chi Minh City, Vietnam).

Design of experiment Two-step and one-step extraction experiments were evaluated according to an experimental design objective of response surface modeling (RSM). A central composite face centered design was used with process modeling and optimization using multiple linear regression modeling. The number of different parameter combinations in each design was 14 with 3 replications of the center point. The varying factors were as follows: acid concentrations, 0, 0.1, and 0.2 M of HCl; extraction temperatures, 30, 60, and 90°C; and reaction times were 1, 3 and 5 h. All extraction experiments were performed in duplicate.

General extraction process Sargassum sp. was ground by milling using an OBH Nordica Coffee Mill 100 watts (OBH Nordica Denmark A/S, Denmark) to pass through a 500-μm sieve and pretreated with a MeOH-$CHCl_3$-H_2O (4:2:1) mixture at room temperature to remove colored matter and phenol compounds prior to extraction. All extractions (Fig. 1a and b) were done in a water bath (Julabo SW22—Germany) with shaking at 200 rpm. 20 mL (HCl) of different concentration (according to the experimental design) were added to a

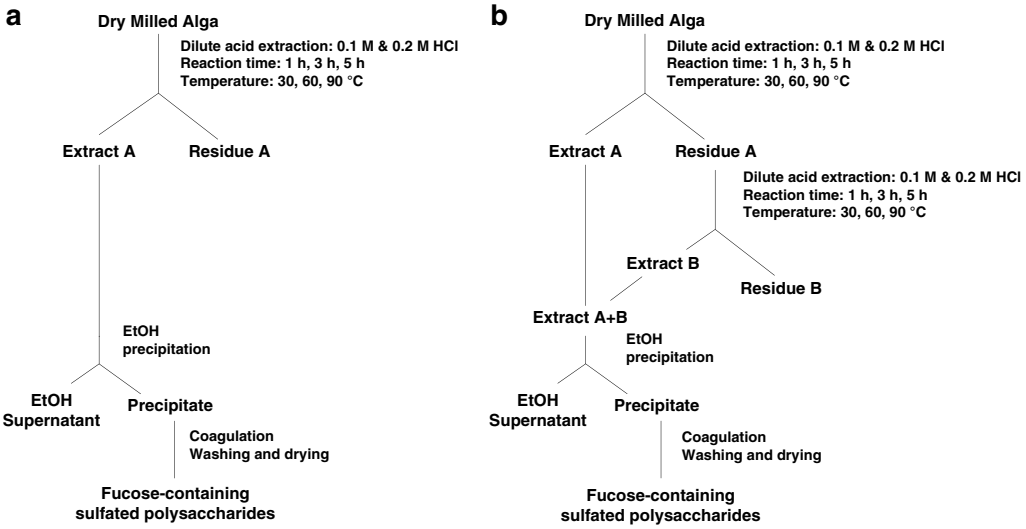

Fig. 1 Schematic diagram of a single-step (**a**) and two-step extraction (**b**) of fucose-containing sulfated polysaccharides from *Sargassum* sp.

centrifuge flask that contained 1 g of ground dried, pretreated *Sargassum* sp. In the single-step extraction (Fig. 1a), the seaweed was extracted according to the factorial design described in the design of experiment. After extraction, the suspended seaweed was centrifuged at 10,600×*g* for 5 min, the supernatant was removed. Then the residue was washed with 5 mL MilliQ water and centrifuged again at 10,600×*g* for 10 min, thereafter the first supernatant and the supernatant from the washing were combined (Extract A). A liquid fraction (16 mL) from Extract A was precipitated with 60% (*v*/*v*) aqueous ethanol to obtain crude FCSPs. The precipitate was washed once with water and was coagulated immediately with either 0.5 M NaCl or 1 M $CaCl_2$ to release and precipitate alginate. The sticky precipitate was discarded and the supernatant was centrifuged again at 10,600×*g* for 10 min. The pellet was collected and transferred to an Eppendorf tube. Each Eppendorf tube was weighed prior to pellet transfer, and then centrifuged at 10,600×*g* for 5 min; the pellet was washed with alcohol and diethyl ether and then finally dried overnight at 50°C. The amount of dried crude FCSPs yield was translated to dry weight (in mg) of the total extract volume (in mL) from the original dry seaweed solution (in grams dry weight per milliliter) and the FCSPs yield (in mg g^{-1} dry weight) was thus based on the original seaweed dry weight. For the two-step extraction (Fig. 1b), the seaweed residue of the single-step extraction (Residue A, Fig. 1b) was extracted once more by addition of 10 mL HCl (of a concentration according to the experimental design) and the suspended seaweed was treated the same way as described above to obtain Extract B. Extract A and B were then combined, and crude FCSPs was isolated by the same procedure as described above (Fig. 1b).

Benchmark experiment A comparative study between the optimized conditions of the two-step extraction and the one-step extraction respectively, obtained from the multivariate models, and two other extraction conditions from known methods (Black et al. 1952; Bilan et al. 2002) was carried out: The *Sargassum* sp. was pretreated as mentioned above prior to extraction. The RSM optimized two-step extraction condition, 0.07 M HCl at 90°C for 3 h (twice), was designated as Method 1. The RSM optimized one-step extraction condition, 0.03 M HCl at 90°C for 4 h (once), or 1 h (once), was designated as Method 1a. Method 2 was the benchmark extraction of Bilan et al. (2002) which, in short, involved extraction of the ground, dried, pretreated *Sargassum* sp. four times with a 2% $CaCl_2$ solution at 85°C for 5 h (Bilan et al. 2002). Method 3 was the benchmark extraction of Black et al. (1952): for this extraction, the ground, dried, pretreated *Sargassum* sp. was extracted three times with 0.17 M HCl at 70°C for 1 h with washing, centrifugation and pH reduction from pH 2.5 to 1.9 after each extraction step (Black et al. 1952). After extraction, all extracts were precipitated with ethanol, washed with H_2O and treated with 1 M $CaCl_2$ to precipitate alginate. In each case, the supernatant was then centrifuged, precipitated with ethanol, centrifuged again, and the pellet was dried overnight at 50°C.

Acid hydrolysis Dried *Sargassum* sp. powder and extracted polysaccharide samples (50 mg) were subjected to acid hydrolysis using 2 M TFA at 121°C for 2 h (Arnous and Meyer 2008). After hydrolysis, the mixture was freeze dried, and the dried powder was resolubilized and centrifuged at 10,000×*g* for 10 min to collect the supernatant then filtered using a 0.2 μm syringe tip filter (Sun Sr. USA) prior to HPAEC-PAD analysis (see below). The monosaccharide recoveries were determined for arabinose, rhamnose, fucose, galactose, glucose, xylose, and glucuronic acid and used as correction factors for the quantitative monosaccharide assessment principally as described previously (Arnous and Meyer 2008).

Compositional analysis The separation and quantification of monosaccharides of the acid hydrolyzed polysaccharides were done by HPAEC-PAD using a BioLC system consisting of GS50 gradients pumps/ED50 electrochemical detector/AS50 chromatography compartment coupled to an AS50 autosampler (Dionex Corp., USA). Separations were performed using a CarboPacTM PA20 (3 mm×150 mm) analytical column (Dionex Corp.) according to Thomassen and Meyer (2010). The quantification was carried out using the external monosaccharide standards: L-arabinose, L-rhamnose, L-fucose, D-galactose, D-glucose, D-mannose, D-xylose and D-glucuronic acid. Data were collected and analyzed with Chromeleon 6.80 SP4 Build 2361 software (Dionex Corp.). The sulfate analysis was performed by a turbidometric method using agarose-barium reagent as described by Jackson and McCandless (1978).

Statistical and data analysis The analyses of variances were performed using Minitab 15 (Minitab, Inc., UK) with a significance value of $P \leq 0.05$. The program Modde version 7.0.0.1 (Umetrics AB, Sweden) was used as an aid for the design of experimental templates and for the evaluation of the effects and the interactions by multiple linear regression analysis.

Results

Polysaccharide compositional profile

The HPAEC analysis of the TFA hydrolysate of *Sargassum* sp. showed that the polysaccharide profile was dominated by fucose (29±3.7), galactose (14±2.1), and glucuronic acid (39±3.5)mg·g^{-1} seaweed dry weight, with a sulfate content of (155±5)mg·g^{-1} seaweed dry weight, while other minor components such as mannose, rhamnose, glucose, arabinose, and xylose were also detected (data not shown). This composition is in accordance with early studies of sulfated polysaccharides from *S. linifolium* (Abdel-Fattah et al. 1974) and typical for the Sargassaceae.

Evaluation of extraction—two steps

Optimizing two-step extraction To determine the optimal extraction of fucose-containing sulfated polysaccharides, the seaweed was extracted twice and the effects of different treatment factors were tested systematically, i.e., acid, 0 to 0.2 M HCl; temperature, 30 to 90°C; and time, 1 to 5 h (Fig. 1b). According to the model, the predicted maximum yield of 7.1% DW, was achieved using 0.07 M HCl/90°C/3 h extracted twice. The predicted extraction yield obtained when the two-step extraction was done without acid, at 30°C for 1 h produced 2.9±0.8% DW only, while the harshest extraction treatment of 0.2 M/90°C/5 h resulted in a yield of 3.1±0.6% DW from the combined Extract A and B, respectively.

Benchmarking Assessment of the optimal parameters obtained from the model was carried out by performing another extraction experiment using the optimum of the response surface model. Subsequently, a comparative extraction study was also done to verify the feasibility of the model. Two extraction methods were compared to the predicted optimal extraction condition: Method 1 was the predicted maximum optimal condition (i.e., 0.07 M HCl, 90°C, and 3 h-two-step extraction); Method 2 was as described by Bilan et al. (2002); and Method 3 was as described by Black et al. (1952). The data showed that the total yields obtained by Method 1 were higher than predicted by the model, namely ~8.5% DW, and that the total polysaccharide yields with the two-step extractions Method 1 and 2 were similar, but much higher than what was obtained with Method 3 (~5% DW; Fig. 2). The data also showed that the major part of the yield was obtained during the first extraction. The results of these experiments

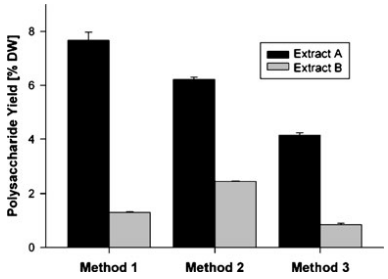

Fig. 2 Polysaccharide yield (% DW) of *Sargassum* sp. extracted using different methods. Method 1 was extracted using 0.07 M HCl/90°C/3 h (two-step); Method 2 as described by Bilan et al. (2002) and Method 3 as described by Black et al. (1952)

thus showed that the yield of the first extraction (Extract A) of Method 1 (7.6±0.31% DW) was higher, $p<0.05$, than the corresponding yields of the benchmark methods Method 2 (6.2±0.08% DW) and Method 3 (4.2±0.09% DW), respectively (Fig. 2). Based on this result, it was decided to evaluate whether a higher polysaccharide yield could be obtained using only a single-step extraction of Method 1 type. A second statistical experiment was therefore performed to define the optimum acid concentration, temperature and incubation time for this single-step extraction (Fig. 1a, Extract A only).

Optimization of polysaccharide yield—single-step extraction

Multiple linear regression analysis of the data obtained in the statistically designed single-step extraction study showed that an increase in extraction time and temperature significantly increased the polysaccharide yield, whereas a decrease of acid concentration also significantly increased the yield (Table 1). Moreover, acid and acid interaction, acid and time interaction and acid and temperature interaction each had a significant effect on the polysaccharide yield (Table 1). According to the model, the maximum polysaccharide yield of ~8% DW from *Sargassum* sp. would be obtained at 0.03 M HCl/90°C/4 h, whereas the lowest polysaccharide yield was at 30°C or 60°C with 0.2 M HCl (Fig. 3).

The quality of the model was confirmed by the average value of the center points (5.76±1.01%DW) being close to the coefficient of the constant (6.28±0.29%DW). The regression model was given as $y=6.28-1.17\ x_1+0.23\ x_2+0.63\ x_3-1.46\ x_1\ x_1-0.35\ x_2\ x_2+0.31\ x_3\ x_3-0.36\ x_1\ x_2-0.51\ x_1\ x_3-0.06\ x_2\ x_3$ (x_1 is acid concentration; x_2 is time; x_3 is temperature; Table 1). The summary of the fit and the predictability of the model for FCSPs yield were satisfactory with $R^2=0.928$; $Q^2=0.853$, model validity=0.891; and model reproducibility=0.889 (Fig. 3).

Table 1 Multiple linear regression results of the parameters and interactions on the polysaccharide yield using the single-step extraction

Parameters and interactions	% DW	
	Coefficient	*P* values
Acid (x_1)	−1.17	3.46×10^{-10}
Time (x_2)	0.23	0.045
Temperature (x_3)	0.63	7.11×10^{-6}
Acid×acid	−1.46	1.96×10^{-7}
Time×time	−0.35	No effect
Temperature×temperature	0.31	No effect
Acid×time	−0.36	0.006
Acid×temperature	−0.51	0.0004
Time×temperature	−0.06	No effect
Constant	6.28	2.56×10^{-22}

Plots of the polysaccharide yields and fucose contents obtained in response to extraction time and temperature further illustrated the effects of the different treatment factors during single-step extraction (Fig. 4). With no acid treatment, the yield of polysaccharide rose when extraction time and temperature increased, while high acid treatment (0.2 M HCl) gave low yield with no significant differences with elevated time and temperature (Fig 4a). After each of the different extraction treatments of the *Sargassum* sp. seaweed, the recovered polysaccharide was sequestered and subjected to TFA hydrolysis and thereafter HPAEC to analyze the monomer content. The results showed that the fucose content was higher in the polysaccharide extracted with 0.2 M HCl than without acid when the treatment time was 1 or 3 h, but at 90°C/5 h, the fucose level was higher with no acid than with 0.2 M HCl (Fig. 4b). The influence of acid treatment (0.2 M HCl) during extraction was thus observed to have a negative effect on the fucose content when time and temperature were elevated (Fig. 4b).

Comparative extraction analysis

The optimal condition of the single-step extraction predicted by the model (i.e., 0.03 M HCl/90°C/4 h) was used in an actual extraction experiment designated as Method 1a for comparative analysis of the monosaccharide composition with the benchmark extraction methods of Bilan et al. (2002): Method 2 and Black et al. (1952): Method 3. The monosaccharide composition of the polysaccharide showed that fucose, galactose, and glucuronic acid were the dominant monomers (Table 2). Clearly, the fucose content of the statistically optimized one-step extraction method was significantly higher than the fucose levels in the fucoidan (or FCSPs) obtained by both Method 2 and 3 (Table 2). Method 2 had the lowest glucuronic acid content among the three methods (Table 2); this was probably due to the interaction of $CaCl_2$ after each round of extraction. The sulfate content of the fucoidan polysaccharide was however lower with the optimized one-step 4-h extraction than that obtained in Method 2 and 3.

Investigation of extended extraction time

Based on the predicted optimized extraction model (Fig. 3) and the raw data (Fig. 4), it was decided to evaluate the influence of extended extraction time on the extracted polysaccharide composition. Extended extraction was carried out for up to 46 h using the predicted one-step optimal

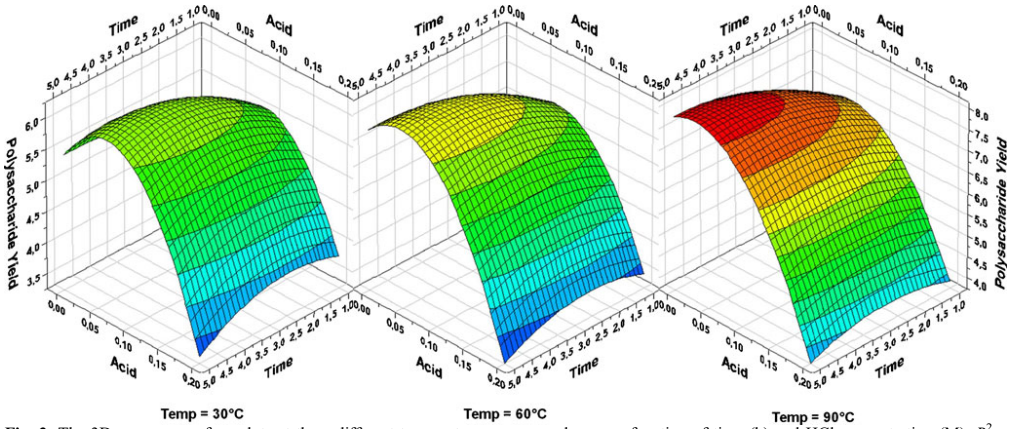

Fig. 3 The 3D response surface plots at three different temperatures each at the optimal extraction condition and maximum fucose-containing polysaccharide yield (% DW) of the single-step extraction procedure as a function of time (h) and HCl concentration (M). R^2= 0.928; Q^2=0.853; model validity=0.891; model reproducibility= 0.889 respectively

treatment, i.e., 0.03 M HCl/90°C. The duration of extraction time influenced the polysaccharide yield (Fig. 5a). Hence, the total polysaccharide yield increased until 8 h of extraction (using 0.03 M HCl/90°C) and the yield then reached a plateau of approximately 9% DW (Fig. 5a). The amount of fucose dropped steadily as the duration of the extraction time increased whereas glucuronic acid increased (Fig. 5a). Apparently, the duration of extraction time also resulted in an almost linear increase in the mannose and galactose content of the extracted polysaccharide, but did not affect the xylose, glucose, and rhamnose contents (Fig. 5b). In addition, the extended extraction time significantly decreased the sulfate content until 8 h of extraction (Fig. 5b). These results indicated that obtaining a high yield of a fucose-rich fucans from *Sargassum* sp. having a limited glucuronic acid (and galactose and mannose) content was a compromise, and the data confirmed that an extraction time of 3 h was the best compromise to achieve high yields and a high fucose level (Fig. 5a). It is tempting to speculate that some "true" fucoidan is released in the early minutes of the extraction, that the acid catalyzed loss of α-fucosyl linkages relative to the other glycosidic bonds during extended treatment may confound the picture. One could interpret the evidence as indicating that the product isolated contained a sulfated fucose-containing heteroglycan, possibly having a glucuronan primary structure with extensive fucosyl side branches which are cleaved and lost as extraction time is extended. A sulfated fucan (a fucoidan) may occur in *Sargassum* sp., but further elucidation of fucose-containing sulfated polysaccharides from *Sargassum* sp. is required. With a lower total polysaccharide yield requirement, a relatively higher fucose content could be obtained with a shorter extraction of 1 h as compared to a 4 h extraction (Table 2 and Fig. 5).

Discussion

Statistically designed optimization of FCSPs extraction was conducted to produce a model that provided an understanding of the complex influences and interactions of the extraction factors temperature, time, and acid concentration, and in turn allowed prediction of the optimal extraction treatment to obtain high FCSPs yield. The model predicted that 0.07 M HCl/90°C/3 h produced high FCSPs yield using two-step extractions. However, careful assessment of the data obtained after the first and second steps at these optimal extraction conditions showed that an additional extraction step to produce Extract B (Fig. 1b) could be omitted since its yield was very low compared to Extract A (Fig. 2). Hence, a new optimized extraction design was performed for a single-step extraction using the same factors settings, i.e., acid, 0 to 0.2 M HCl; temperature, 30 to 90°C; and time, 1 to 5 h. The maximum yield produced by this new optimal condition (0.03 M HCl/90°C/ 4 h) was ~7.0% DW. Previously reported multi-step extraction results of fucose-containing polysaccharides from *S. horneri* and *U. pinnatifida* were 5.17% DW and 6.77–15.10% DW (Kuda et al. 2002); from *S. ringgoldianum* it was 200 mg from fresh 150 g algal fronds, approximately equivalent to 0.67% DW (Mori and Nisizawa 1982). Hence, the result of multiple linear regressions of the parameters and interaction on the fucose-containing poly-

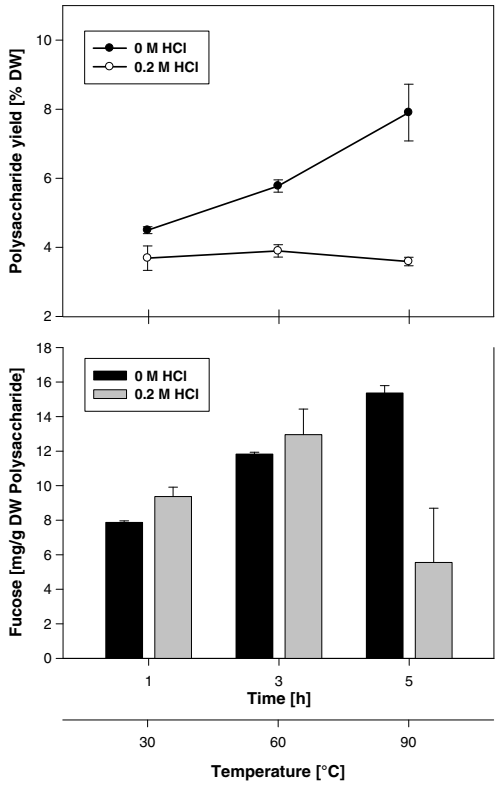

Fig. 4 Polysaccharide yield (**a**) and fucose content (**b**) of different combination of extraction treatment (i.e., acid, time and temperature) of fucose-containing polysaccharide seaweed (*Sargassum* sp.). Data given as mean±SD, $n=3$

saccharide yield for the one-step extraction was in agreement with the available data.

The composition obtained from HPAEC-PAD analysis of the TFA hydrolysate of *Sargassum* sp. agrees with the prevalent polysaccharide structure among *Sargassum* species; hence, in the case of *S. patens* and *S. stenophyllum* (Duarte et al. 2001; Zhu et al. 2003) the structure has been found to be a linear backbone of (1→6)-β-D-galactose and/or (1→2)-β-D-mannose units with branched chains formed by (1→3) and/or (1→4)-α-L-fucose, (1→4)-α-D-glucuronic acid, terminal β-D-xylose and sometimes (1→4)-α-D-glucose. However, as pointed out by Percival and McDowell (1967): "Fucoidin, first isolated and named by Kylin (1913) was more systematically named fucoidan". The algal source in this case was *F. vesiculosus*, and the Kylin product has been established as a fucan sulfate (currently termed fucoidan as it was isolated from brown

Table 2 Analysis of monosaccharide after 2 M TFA hydrolysis of the sulfated polysaccharide fractions of *Sargassum* sp. isolated by different extraction methods

Monomers (mg/g DW)	Method 1a (1 step—1 h)	Method 1a (1 step—4 h)	Method 2	Method 3
Fucose	113±0.9	97±2	49.2±1.7	54.8±1.7
Rhamnose	6.2±0.6	2.9±0.3	6.29±0.07	nd
Galactose	39±6	39 ±1.9	25.8±0.30	36±1.34
Glucose	11.1±0.2	3.7±0.4	10.9±0.13	13.7±1.25
Mannose	11.3±1.6	11.1±1.7	2.41±0.03	5.76±0.21
Xylose	15.7±1.1	11.4±1.4	5.12±2.36	6.8±1.7
Gluc Acid	85±11.7	97±2.7	10.0±0.12	45±5.8
Sulfate	362±16.5	308±11	341±5.2	327±8.5

Data given as average values±standard deviation, $n=3$. *Method 1a* extraction by using the optimized model of the one-step extraction (0.03 M HCl/90°C) with an extraction time of either 1 h or 4 h as indicated. *Method 2* as described by Bilan et al. (2002), and *Method 3* as described by Black et al. (1952). General linear model significantly different ($P<0.05$)

seaweed). Percival and McDowell understood and emphasized that fucoidan referred to polysaccharides consisting almost entirely of fucose and ester sulfate. Fucose-containing heteropolysaccharides (e.g., glucuronoxylofucans) were treated as quite different entities from fucoidan. Painter (1983) provided the following definition: "Natural polysaccharides built up essentially of sulfated α-L-fucose residues are known as fucoidans". This working definition of fucoidan has been retained by polysaccharide chemists to the present (Mabeau et al. 1990; Chevolot et al. 1999; Berteau and Mulloy 2003).

The optimization of the one-step method illustrated that a single-step extraction with the combination of low acid concentration, 0.03 M HCl or below, and temperature near 90°C was sufficient to produce a satisfactory FCSPs (fucoidan) yield. The integrity of the polysaccharide was best conserved at low acid treatment (Fig. 4b), since the use of 0.2 M HCl apparently broke the integrity of the polysaccharide molecules resulting in a decline of fucose at elevated time and temperature (Fig. 4b). This indicated that the higher acid levels might have caused a loosening of the cell wall matrix allowing local penetration of the acid into the fucoidan in the intercellular spaces resulting in partial degradation of FCSPs or notably fucoidan (Kloareg 1984; Mabeau et al. 1990). The effect of acid might have been enhanced as dried seaweed material was used in the experiment, where it can absorb and expand abruptly during hydration (Phillips et al. 2002). The degradation of carbohydrate chains built up of fucose was recognized when the duration of extraction time increased (Fig. 5). Our result is in agreement with Ponce et al. (2003) who reported that longer extraction time led to poorer fucose content. In

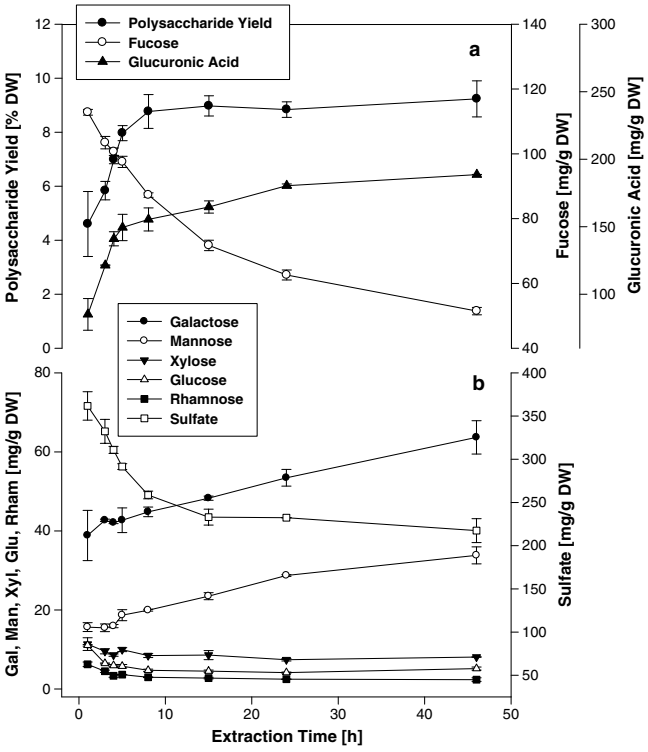

Fig. 5 Polysaccharide yield and monosaccharide content as a result of prolonged reaction time using the predicted optimized one-step extraction condition 0.03 M HCl/90°C. **a** Polysaccharide yield, fucose and glucuronic acid, **b** galactose, mannose, xylose, glucose, rhamnose and sulfate

addition, longer extraction time at higher temperatures led to higher polysaccharide yield with lower amount of sulfate and higher proportion of glucuronic acid (Ponce et al. 2003; Duarte et al. 2001). The same trend was also noticed in our present work. The glucuronic acid increased while sulfate decreased with time, i.e., the sulfate content was highest when glucuronic acid was lowest (Fig. 5a, b). This was also observed for fucoidan extraction from *S. ringgoldianum* (Mori and Nisizawa 1982).

In conclusion, a simple and practical method for recovering a suite of complex fucose-containing sulfated polysaccharides from *Sargassum* sp., has been established. Clearly, yield and chemical composition of the product are strongly affected by the method of extraction as was to be expected. The yield data are gravimetric only and so pertain to a crude mixture of biopolymers extracted. The evidence presented shows that the extracted polysaccharide product is heterogeneous at any time it is analyzed, although the composition varies with the duration of extraction. The monomeric composition shows that fucose and sulfate were important components of the polysaccharide mixture as isolated. An optimized one-step extraction treatment to obtain high yields of a fucose-containing sulfated polysaccharide from *Sargassum* sp. was developed, and the effect of different treatment parameters on the integrity of the polysaccharide was established. The results confirmed that *Sargassum* sp. may be a good source of fucose-containing sulfated polysaccharides. The data also demonstrated the vulnerability of fucose-containing sulfated polysaccharides to harsh extraction conditions and confirmed that the extraction method significantly influences the yields and not least the polysaccharide composition of the extracted polysaccharide. Furthermore, the main conclusions confirm the long known facts that cell wall polymers of brown algae are complex, and that the yields and chemical nature of polysaccharides recovered from such seaweeds are markedly influenced by the conditions used to extract them. It is important to emphasize this point as it has a major bearing on any study in which such products are being evaluated for biological activity. It is our belief that the model obtained may be applied to other FCSPs or fucoidan-containing types of brown seaweed.

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4 Bioactivity of FCSPs

Fucoidan was first isolated in 1913; since then, it has gained much attention in both the academic and industrial sectors (Jiao et al., 2011, Kylin, 1913). Fucoidan has been the subject of many research studies due to its diverse biological functions, including anti-tumor and immunomodulatory activities (Alekseyenko et al., 2007; Maruyama et al., 2006). According to the ISI Web of Knowledge (Thomson Reuters), the number of published articles has increased significantly since fucoidan, or "fucoidin" as it was first called, was first isolated from brown algae in 1913 (Kylin, 1913); in particular, a profound increase in the number of papers has occurred over the last 5–10 years. By now, the published papers related to fucoidan hit approximately 1,800 (August 2011, Fig. 4.1). Recent interests have focused mainly on the potentially beneficial biological activities of fucoidan and FCSPs in humans, including anti-tumor, immunomodulatory, anti-inflammatory, antiviral, antithrombotic, anticoagulant, and antioxidant effects as well as specific activities against kidney, liver, and urinary system disorders. Interest in utilizing natural bioactive compounds for the suppression or prevention of cancer is flourishing because of the current development of approaches has been recognized as a field with enormous potential (Rahman et al., 2010).

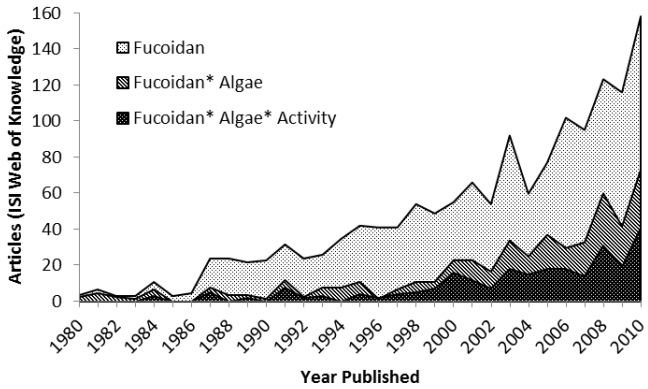

Fig. 4.1. The trend over 3 decades of research on fucoidan as depicted by the number of articles published annually (Thomson Reuters, ISI Web of Knowledge). The number of articles was obtained according to topics being assigned in ISI Web of Knowledge search engine with the following topic search terms: fucoidan; fucoidan*algae; fucoidan*algae*activity.

While the development of research efforts involving FCSPs and their potential applications continue to advance, understanding of the mechanisms and the particular structural features of the FCSPs being responsible for the various biological activities remains incomplete. Seaweeds, including various brown seaweeds such as *Undaria* and *Laminaria* spp., are part of the food culture in Asia, notably in Japan, the Philippines, and Korea, and seaweed extracts have also been used as remedies in traditional medicine. However, there currently exist no standardized FCSPs extraction or purification protocols, and no specific pharmaceutical, dermatological, or nutraceutical applications have as yet been officially approved for these polysaccharides or their lower molar mass oligosaccharide derivatives. FCSPs that were isolated in multi-step processes and then purified and fractionated demonstrated essential bioactivities (Holtkamp et al., 2009), while unpurified fucoidan that is isolated using milder and fewer processing step has been found to induce anti-tumor activity and act as an immunopotentiator in tumor-bearing animals (Takahashi, 1983).

4.1 Anti-tumor activity of FCSPs

FCSPs from different brown seaweed species have shown remarkable growth inhibition of Sarcoma-180 cells implanted into mice and possess anti-tumor activity against L-1210 leukemia in mice (Yamamoto et al., 1974, 1981, 1984). The anti-tumor mechanism of fucoidan from sporophyll of *Undaria pinnatifida* was described by Maruyama et al. (2003), who indicated that the anti-tumor activity of fucoidan appears to be associated with significant enhancement of the cytolytic activity of NK cells. The effectiveness of fucoidan as an immunopotentiator was exhibited by an increased immune response against A20 leukemia cells and a significantly lowered tumor size in transgenic (DO-11-10-Tg) mice (Maruyama et al., 2006). The enhancement of NK cell activity by fucoidan was augmented through increase production of macrophage-mediated immune responses, namely IL-2, IFN-γ, and IL-12 (Maruyama et al., 2003). Moreover, the most prevalent pathway through which fucoidan can inhibit cancer growth is apoptosis. Fucoidan induces apoptosis via the activation of caspase-3 in human HS-Sultan cells (Aisa et al., 2005); in MCF-7 cells via caspase-8–dependent pathways (Yamasaki-Miyamoto et al., 2009); and through the activation of caspases via both the death receptor-mediated and the mitochondria-mediated apoptotic pathways (Kim et al., 2010).

4.2 Anti-proliferative and immune-response activities

This section is an extended elucidation of Paper 3: fucoidan from *Sargassum* sp. and *F. vesiculosus* reduces cell viability of lung carcinoma and melanoma cells *in vitro* and activates NK cells in mice *in*

vivo. In addition, highlights of Paper 4: FCSPs inhibit the proliferation of melanoma cells and induce apoptosis by activating caspase-3 *in vitro* will also be presented in this section.

4.2.1 Relevance

Lung cancer is among the most prevalent types of cancer worldwide and is a prime contributor to cancer-related mortality. Melanoma incidence rates for both males and females are also increasing in the United States (Jemal et al., 2010). To date, therapeutic strategies such as chemotherapy, radiation therapy, surgery, or combinations thereof have been implemented for many cancer patients; however, they still provide only minimal survival benefits due to factors such as toxicity, complications, and long-term side effects (Schneider et al., 2010; Grossi et al., 2010). As a consequence, the need for chemopreventive agents from natural sources with minimal or no harmful side effects is of ardent importance. Hence, FCSPs, notably fucoidan, from brown seaweed may prove to be excellent contenders for the prevention or control of lung and skin carcinogenesis.

4.2.2 Hypotheses and objectives

Isolating fucoidan from brown seaweed using minimal processing will preserve its structural integrity and, thereby, help maintain its bioactive characteristics but results in crude fucoidan products. The chemical nature of polysaccharides recovered from seaweed is influenced by the technology used to extract them. Crude fucoidan obtained via single-step extraction has been subjected to fewer and milder process conditions. Therefore, the well-defined structural features of fucoidans are likely be conserved and, thus, retain their biological activity.

It has been reported that crude fucoidan fractions from edible brown seaweeds affect L-1210 leukemia cell development *in vivo*, while sulfation of crude fucoidan fractions from *Sargassum kjellmanianum* enhances their anti-tumor activity (Yamamoto et al., 1983). Moreover, *in vivo* studies confirmed that the feeding of ground brown seaweed to animals, the oral administration of hot-water extraction of seaweed, and the intraperitoneal injection of crude fucoidan fractions resulted in an inhibitory effect on mammary tumorigenesis and intestinal carcinogenesis (Yamamoto et al., 1987; Yamamoto and Maruyama, 1985). Hence, other forms of cancer such as lung and skin cancer may alternatively be prevented or controlled through the use of crude fucoidan from brown seaweed.

Therefore, the objectives of the present works were to determine the potency of unfractionated FCSPs to inhibit the growth of skin and lung cancer cells *in vitro*, evaluate the immune-response

activity of FCSPs in mice *in vivo*, and elucidate the contributing factors behind this effect (Paper 3). Furthermore, we investigated the different structural features of unfractionated FCSPs from *Sargassum* sp. and *F. vesiculosus* using Fourier transform infrared (FT-IR) and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy to determine whether its contribution is crucial to its bioactive effectiveness. We also conducted an *in vitro* study to examine the influence of FCSP products from *Sargassum* sp. and *F. vesiculosus* on melanoma B16 cell (MC) proliferation and caspase-3 activity mediating the apoptosis of melanoma B16 cells (Paper 4).

4.2.3 Result highlights

The influence of 2 crude fucoidans extracted from *Sargassum* sp. (MTA) using a minimal number of processing steps and obtained commercially from *F. vesiculosus* (SIG) on Lewis lung carcinoma cells (LCC) and MC was examined. The compositions of the SIG and MTA fucoidans were significantly different with respect to fucose, galactose, and glucuronic acid, unlike the sulfate content (Paper 3). The FT-IR spectra indicated that the sulfate in the FCSPs from *Sargassum* sp. (FSAR) was located in the equatorial C-2 and/or C-3 positions as depicted by the absorption bands at 817 cm^{-1}, whereas the IR spectra of FCSPs from *F. vesiculosus* (FVES) displayed an absorption band at 838 cm^{-1} with a small shoulder absorption band at 822 cm^{-1}, indicating sulfate groups at the C-4 and C-2 positions (Paper 4). This finding corresponds to the ^{1}H NMR spectra of the unfractionated FVES sample from *F. vesiculosus,* indicating a typical structure of algal fucoidan consisting of α3-linked 2-mono-*O*-sulfated L-fucopyranose residues and/or α3-linked 2,4-di-*O*-sulfated L-fucopyranose residues (Pereira et al., 1999; Patankar et al., 1993).

In vitro studies showed the anti-proliferative effect of crude fucoidan on LCC and MC cells in a dose-dependent manner. Male C57BL/6JJCL mice were subjected to daily intraperitoneal injections over 4 days with either SIG or MTA fucoidan (50 mg/kg body wt) to evaluate immune response augmentation. The cytolytic activity of NK cells was enhanced by crude fucoidan as indicated by ^{51}Cr-labeled YAC-1 target cell release. Histochemical staining showed morphologic changes of MC cells after exposure to crude fucoidan. Fragmentation and condensation of chromatin, illustrated as an intense dark brown color within the cell nuclei, was indicative of crude fucoidan-induced apoptosis (Paper 3). In this work (Paper 4), we noted based on flow cytometric analysis that FSCP samples from *Sargassum* sp. and *F. vesiculosus* induced apoptosis through activation of caspase-3 in a dose-dependent manner (Paper 4).

The mechanism behind FCSP anti-tumor activity and how it enhances the immune response has yet to be determined. Nevertheless, this study provides substantial indications that FCSP exerts

bioactive characteristics on lung and skin cancer model cells and that its anti-tumor activity was due to the enhancement of NK cell activity (Paper 3). The crucial bioactive effectiveness of these unfractionated FCSPs from *Sargassum* sp. and *F. vesiculosus* may be attributed to their distinct structural features, such as level of sulfation (charge density) and position and bonding of the sulfate substitutions or sulfated fucans and sulfated galactan complexes. This present study demonstrated the early and later apoptosis stages by FACScan, which could indicate that FCSPs have a direct apoptotic effect on MC cells (Paper 4) and that the presence of 3-linked galactan in the structure of FCSPs from *Sargassum* sp. may somehow contribute to its anti-proliferative effects.

4.2.4 Consideration and justification

The monosaccharide profiles of the acid-hydrolyzed polysaccharides from *Sargassum* sp. and *F. vesiculosus* suggest that both products are contaminated with components other than fucose and sulfate (Paper 3). The commercially obtained sample from *F. vesiculosus* was a crude fucoidan, as indicated in the product specification from the supplier (Sigma Aldrich Inc., Germany). Alternatively, the product extracted from *Sargassum* sp. contained a low amount of fucose compare to that of *F. vesiculosus*, but they have the same chemical composition profile. The low amount of recovered fucose was probably attributed by the condition used to extract them or the extent of the acid hydrolysis, which perhaps has a major influence in the disappearance of fucose. In any case, the isolated product from *Sargassum* sp. showed the same monosaccharide profile as the commercially obtained sample from *F. vesiculosus* (Sigma Aldrich Inc.); hence, it is safe to say that the isolated FCSP product from *Sargassum* sp. was also a crude fucoidan.

Moreover, based on previous published papers, the term crude fucoidan was applied for FCSPs from brown seaweeds in which impurities are present in the isolated products (Yamamoto et al., 1983; Takahashi, 1983). Nevertheless, crude fucoidan from these samples have shown potent anti-tumor activity and cellular immunity associated with T cells (Takahashi, 1983). However, it should be noted that evidence establishing that fucose comprised the backbone of a significant component of the preparation was necessary to reasonably apply the term fucoidan. In the absence of this evidence, it is preferable to term the isolated product from *Sargassum* sp. as crude fucoidan or, to be more accurate, FCSP (Papers 3 and 4). The main active component may be a FCSP (e.g., fucoidan) that is present in brown seaweed such as *Sargassum* sp., but further structural elucidation is essential, as it has major bearing on any biological activity study. Nevertheless, there are many indications in this present work that FCSP samples may contain

fucoidan-like structures (Paper 4). This study showed that both FCSP samples induced apoptosis by activating caspases-3 and exerted anti-tumor activity by inhibiting the growth of MC cells. It appears that unfractionated FCSPs from *Sargassum* sp. and *F. vesiculosus* are potent skin cancer preventive agents, a fact that was demonstrated by a direct *in vitro* study of melanoma B16 cells (Paper 4).

4.3 Paper 3: Fucoidan from *Sargassum* sp. and *Fucus vesiculosus* reduces cell viability of lung carcinoma and melanoma cells *in vitro* and activates natural killer cells in mice *in vivo*

International Journal of Biological Macromolecules, 2011, **in Press**

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4.4 Paper 4: Fucose containing sulfated polysaccharides inhibits the proliferation of melanoma cells and induces apoptosis by activation of caspase-3 *in vitro*

Marine Drugs, 2011, **Submitted**

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Fucoidan from *Sargassum* sp. and *Fucus vesiculosus* reduces cell viability of lung carcinoma and melanoma cells *in vitro* and activates natural killer cells in mice *in vivo*

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ABSTRACT

Fucoidan is known to exhibit crucial biological activities, including anti-tumor activity. In this study, we examined the influence of crude fucoidan extracted from *Sargassum* sp. (MTA) and *Fucus vesiculosus* (SIG) on Lewis lung carcinoma cells (LCC) and melanoma B16 cells (MC). *In vitro* studies were performed using cell viability analysis and showed that SIG and MTA fucoidans significantly decreased the viable number of LCC and MC cells in a dose–response fashion. Histochemical staining showed morphological changes of melanoma B16 cells after exposure to fucoidan. The observed changes were indicative of crude fucoidan induced apoptosis. Male C57BL/6JJCL mice were subjected to daily *i.p.* injections over 4 days with either SIG or MTA fucoidan (50 mg/kg body wt.). The cytolytic activity of natural killer (NK) cells was enhanced by crude fucoidan in a dose-dependent manner as indicated by ^{51}Cr labeled YAC-1 target cell release. This study provides substantial indications that crude fucoidan exerts bioactive effects on lung and skin cancer model cells *in vitro* and induces enhanced natural killer cell activity in mice *in vivo*.

1. Introduction

Fucoidan is a term used for a group of fucose-rich sulfated polysaccharides containing varying amounts of galactose, xylose and glucuronic acid. Fucoidan can be extracted from brown seaweeds like *Sargassum* sp. and *Fucus* sp. Recently, the diverse biological activities of fucoidan have been studied intensively; the putative bioactivities of fucoidan include antitumor and immunomodulatory [1,2], antivirus [3], antithrombotic and anticoagulant effects [4]. The utilization of natural bioactive compounds for suppression or prevention of cancer is thriving and has been recognized as a field of enormous potential [5]. Fucoidan extracted from brown seaweeds has been reported to enhance the activity of NK (natural killer) cells which is an important factor in anti-cancer activity [2]. Fucoidan has also been shown to induce a substantial reduction in viable cell numbers and apoptosis of human HS-Sultan cells as well as HT-29 and HCT116 cells in a dose-dependent manner [6,7].

Fucoidans can be extracted and purified from seaweeds via various multi-step processes involving chemical, physical and/or enzymatic treatments and different purification and fractionation steps [8]. Although differently extracted and modified fucoidans have been reported to exert bioactivity, crude fucoidan has been found to exert immunopotentiating effects in tumor bearing animals, leading to anti-tumor effectiveness [9]. Crude fucoidan (or fucose-containing sulfated polysaccharides) can be obtained through dilute acid or aqueous extraction using milder and fewer processing steps that induce minimal structural alterations thereby maintaining the natural characteristics of fucoidan. To the best of our knowledge, the effect of crude fucoidan on lung and skin cancer cells and immune-response activity has yet to be determined. Therefore we examined the effects of crude fucoidan extracted from *Sargassum* sp. and *Fucus vesiculosus* on the proliferation of Lewis lung carcinoma and melanoma B16 cells and evaluated its influence on immune response activity.

The principal objectives of the present work were thus to determine whether crude fucoidan can inhibit the growth of skin and lung cancer cells, and if so, to describe the contributing factors behind this effect. In this study, we determined that crude fucoidan hinders the *in vitro* growth of Lewis lung carcinoma and melanoma B16 cells by induction of apoptosis. Moreover, the anti-tumor activity of crude fucoidan seems to be associated with an enhanced immune-response, as depicted by an increase in NK cell activity in mice.

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2. Materials and methods

2.1. Chemicals

Two crude fucoidan samples were used throughout this work. Firstly, crude fucoidan from *Sargassum* sp. (MTA) was extracted in our laboratory (see Section 2.2) while crude fucoidan from *F. vesiculosus* (SIG) was obtained commercially (Sigma–Aldrich Co., Steinheim, Germany). Dried *Sargassum* sp. was obtained from Viet Delta Ltd. (Ho Chi Minh, Vietnam). Minimal essential medium eagle (MEM-eagle) cell culture media was purchased from Sigma–Aldrich Co. (Steinheim, Germany); foetal bovine serum (FBS) was from Flow Laboratories (North Ryde, N.S.W., Australia); streptomycin–penicillin and Trypan Blue were from Gibco (Canada). Hydrochloric acid (37%), D-glucose and D-xylose were purchased from Merck (Darmstadt, Germany). Trifluoracetic acid (99%, TFA), trichloroacetic acid (99%, TCA), $CaCl_2$, Na_2SO_4, $BaCl_2$, arabinose, rhamnose, D-galactose and L-fucose were from Sigma–Aldrich Co. (Steinheim, Germany). Agarose D-2 was obtained from Hispanagar (Burgos, Spain). All chemicals used were analytical grade.

2.2. Extraction of crude fucoidan (MTA)

Seaweed from *Sargassum* sp. was ground and sieved to pass through a 500 μm sieve. Crude fucoidan extraction was carried out by adding 100 g of dried ground seaweed to a 5 L flask containing 2 L of 0.03 M HCl. The mixture was then placed in a 90 °C water bath (Julabo, Germany) with continuous mixing at 200 rpm for 4 h. The suspended seaweed was filtered (Whatman filter paper, no. 1) to obtain a fucoidan extract. The extract was then precipitated using 60% ethanol (EtOH), and the precipitate was collected after centrifugation (Sigma Laboratory Centrifuge 4K15, VWR, Denmark) at 10,600 rpm for 10 min, and the resulting pellet was freeze dried. This pellet was the crude fucoidan and is referred to as MTA in this study. The crude fucoidan (SIG) derived from *F. vesiculosus* was commercially obtained from Sigma–Aldrich Co. (Steinheim, Germany). According to the product description the *F. vesiculosus* fucoidan had been prepared via the extraction method described by Black [10].

2.3. Acid hydrolysis

The freeze dried crude fucoidan powder (MTA, 20 mg) and the commercially obtained crude fucoidan (SIG, 20 mg) were hydrolyzed separately using 2 M TFA (final concentration) at 121 °C for 2 h [11]. The hydrolyzed mixtures were then freeze dried at −57 °C (Heto Lyolab 3000, England). The dried powders were resolubilized in doubly distilled water and centrifuged at 10,000 rpm for 10 min to collect the supernatants. Each supernatant was filtered through a 0.2 μm syringe tip filter (SUN-SRi, Rockwood, TN) prior to injection into the HPAEC-PAD for monosaccharide analysis (see Section 2.4).

2.4. Fucoidan composition analysis

The supernatant of each of the acid hydrolyzed crude fucoidan samples was analyzed for monosaccharides and sulfate contents. The separation and quantification of monosaccharides were performed by HPAEC-PAD analysis using an ICS-3000 system consisting of gradient pumps (model DP-1), an electrochemical detector/chromatography module (model DC-1) and an autosampler (Dionex Corp., Sunnyvale, CA). Separation was achieved using a CarboPac^{TM} PA20 (3 mm × 150 mm) analytical column following the method described by Arnous and Meyer [11]. Data quantification was carried out using the Chromeleon 6.8 SP4 Build 2361 chromatography software (Dionex Corp., Sunnyvale, CA). Recovery values of the monosaccharides were estimated from parallel runs, i.e., TFA hydrolysis, freeze drying, and HPAEC-PAD analysis, of monosaccharide standards as described previously [11]. Analysis of sulfate content was done according to the method described by Jackson and McCandless [12].

2.5. Cell culture and anti-tumor activity assay

Lewis Lung Carcinoma (LLC) and melanoma B16 cells (MC) were grown in MEM eagle medium supplemented with 10% (v/v) heat-inactivated FBS, 1% streptomycin–penicillin and 1% of 200 mM L-glutamine. The cells were maintained at 37 °C under 5% CO_2. An aliquot (10 μl) of the cell-MEM-FBS medium mixture was diluted with 90 μl of 0.4% Trypan Blue for cell counting. Monolayer cultivation was carried out by adding 100 μl of the cell-MEM-FBS mixture in 96-flat well plates at a density of 3×10^4 cells per well. Plates were then incubated for 24 h in 5% CO_2 at 37 °C. Afterwards the medium was removed and replaced with 100 μl of MEM medium containing 2% FBS and varying concentrations (0.1–1.0 mg/ml) of *Sargassum* sp. crude fucoidan (MTA) and commercial crude fucoidan from *F. vesiculosus* (SIG) and then incubated for 24 h. A 20 μl MTT (5 mg/ml) solution was added to the cultures and they were then re-incubated for 4 h. Finally, 100 μl of stabilization solution was added to each well and the plates were incubated overnight at 37 °C under 5% CO_2. Absorbance was measured using an Elisa reader at 550–690 nm.

2.6. Natural killer (NK) cell activity based on ^{51}Chromium (^{51}Cr) release assay

Male C57BL/6J mice (Clea Japan Inc., Tokyo, Japan) were weighed prior to intraperitoneal injection (*i.p.*) of fucoidan samples (50 mg/kg body wt.) from *Sargassum* sp. (MTA) and commercial fucoidan from *F. vesiculosus* (SIG) in 0.1 ml saline for 4 successive days; neat saline was used as control, and Poly I:C as positive control. 3 mice were used per treatment ($n = 3$). The mouse spleen was removed and suspended in complete RPMI-1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM of L-glutamine and 10% FBS. Spleen cells were seeded in 0.1 ml of this complete medium per well in 96-well microtiter plates (V-typed, Nalge Nunc, Tokyo, Japan) at densities of 1×10^7, 0.5×10^6, and 0.25×10^6 cells/ml. ^{51}Cr (with sodium chromate, 37 MBq/ml, Dupont, NEN Research Products, DE, USA) was used to label YAC-1 cells using an exposure time of 1 h after which the cells were washed, brought to a density of 1×10^4/ml and added in 0.1 ml aliquots to the wells. The plate was centrifuged for 3 min at 800 rpm and room temperature, and then incubated for 4 h in 5% CO_2 at 37 °C. Percent specific lysis was calculated using the formula: % specific ^{51}Cr release = [(mean cpm experimental release − mean cpm spontaneous release)/(mean cpm total releasable counts − mean cpm spontaneous release)] × 100, based on results from radioactivity measurements of the supernatant using a γ-counter (Gamma 5500B, Beckman Instruments Inc., CA, USA). The experiments adhered to the National Institute of Health's guidelines for the use of experimental animals and the experimental protocol was approved by The Animal Use Committee of the Kitasato University School of Allied Health Sciences before the study was initiated.

2.7. Apoptosis detection assay

Both the MTA and SIG crude fucoidan samples (0.8 mg/ml dose concentration) and a control (no fucoidan) were tested on melanoma B16 cells for programmed cell death by the terminal deoxynucleotidyl transferase-mediated deoxyuridine

triphosphate (dUTP) nick end-labeling (TUNEL) method using an *in situ* apoptosis detection kit (Takara Bio Inc., Shiga, Japan). The melanoma B16 cells were grown in a 2-well permanox slide (Lab-Tek Chamber Slides, Nalge Nunc, Tokyo, Japan) at 5×10^4 cells/ml density per slide, treated with fucoidan for 48 h and then washed twice with PBS solution followed by fixing of the cells using 4% paraformaldehyde/PBS (pH 7.4). The cells on the slides were assayed according to the standard protocol provided in the apoptosis detection kit. Apoptotic cells were viewed using a BX51 light microscope (Olympus Corp., Tokyo, Japan).

2.8. Statistical analyses

Tabulation of data and calculation of mean and standard deviations were done using Excel (Microsoft Office 2010). Analysis of variance was performed using Minitab 15 (Minitab Inc., State College, PA, USA) using a significance value of $P \leq 0.05$.

3. Results

3.1. Fucoidan extraction and compositional analysis

The crude MTA fucoidan extracted from *Sargassum* sp. was mainly made up of fucose, glucuronic acid, and sulfate (Table 1). Minor amounts of other monosaccharide constituents such as galactose, glucose, xylose, mannose, rhamnose and arabinose were also detected (Table 1). The SIG fucoidan had a similar monosaccharide pattern, but the amounts of fucose, galactose, and xylose, were significantly higher in the SIG fucoidan, whereas glucuronic acid was significantly lower than in the MTA fucoidan (Table 1). No significant difference was found in the sulfate content of MTA and SIG fucoidan (Table 1).

3.2. Crude fucoidan inhibits skin and lung cancer cell growth in vitro

In vitro assessment of the effects of different concentrations (0–1.0 mg/ml) of crude MTA and SIG fucoidan on the growth of Lewis Lung Carcinoma (LLC) and melanoma B16 (MC) cells was performed via measurement of cell viability using an MTT assay. The growth of LLC and MC cells were affected in a dose-dependent manner following the addition of crude MTA and SIG fucoidan, based on 3×10^4 cells per well density after 24 h of incubation (Fig. 1a and b). MTA and SIG fucoidan performed similarly and reduced the number of viable LLC cells present in a dose-dependent fashion, with cell viabilities reduced to $40 \pm 7\%$ and $36 \pm 14\%$ after addition of 1.0 mg/ml MTA and SIG, respectively (Fig. 1a). A drastic reduction in cell numbers was also noted for the MC cells, resulting in only $56 \pm 5\%$ and $50 \pm 5\%$ viable cells upon addition of 0.1 and 0.2 mg/ml MTA fucoidan, respectively. As such, the reduction in cell viability induced by MTA fucoidan was significantly more pronounced ($P \leq 0.05$) than the cell reduction induced by SIG fucoidan, especially at low fucoidan addition levels (Fig. 1b). At dosage levels of 0.4–1.0 mg/ml, the cell viability of the MC cells was reduced to ~10–55% (lowest cell viability at the higher fucoidan dosage), but no significant differences were observed in the effects of MTA and SIG (Fig. 1b). It may also be observed from Fig. 1 that fucoidan appeared more effective in reducing the number of MC viable cells than LLC cells in a dose-dependent manner, with e.g., only $32 \pm 2\%$ viable MC cells (Fig. 1b) and $57 \pm 7\%$ viable LCC cells (Fig. 1a) at a SIG fucoidan concentration of 0.6 mg/ml.

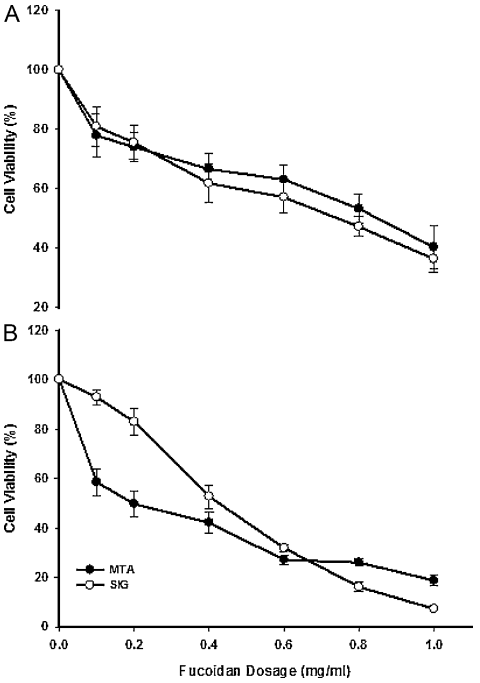

Fig. 1. *In vitro* analysis of the direct activity on carcinoma and melanoma cells of crude fucoidan derived from *Sargassum* sp. (MTA) and commercial fucoidan (SIG) derived from *F. vesiculosus*. (a) Fucoidan activity on Lewis Lung Carcinoma cells, and (b) fucoidan activity on melanoma B16 cells. Cell density was 3×10^4 cells per well. Data are shown as average values ± s.d. Level of significance was $P \leq 0.05$, $n = 4$.

3.3. Crude fucoidan induced apoptosis of melanoma B16 cells

To be able to determine whether crude fucoidan-induced reduction of cell viability was attributed to the initiation of apoptosis, the TUNEL method using melanoma B16 cells and 3,3′ Diaminobenzidine (DAB) substrate with counterstaining using 1% methylgreen was employed. The evaluation of apoptosis by the two types of fucoidan was only done on the melanoma B16 cells because these cells appeared to be particularly sensitive to the MTA fucoidan and exhibited a differential growth response to the two types of crude fucoidan (Fig. 1). The treatment of melanoma B16 cells with

Table 1
Monosaccharide constituents detected by HPAEC-PAD from the TFA hydrolyzed samples of *Sargassum* sp. fucoidan (MTA), and commercial fucoidan (SIG) from *F. vesiculosus*.

Samples	Monosaccharide composition[a] in mg/g DW								
	Fuc*	Rha	Ara	Gal*	Glc	Xyl*	Man*	GluA*	Sul
MTA	31.4 ± 2.0	1.6 ± 0.1	0.2 ± 0.1	13.9 ± 0.8	4.2 ± 0.1	4.2 ± 0.3	5.7 ± 0.5	122.8 ± 7.0	384.4 ± 26.2
SIG	138.7 ± 5.5	2.0 ± 0.6	2.8 ± 0.2	27.9 ± 1.4	2.5 ± 1.8	12.8 ± 1.6	0.2 ± 0.4	18.5 ± 1.9	341.6 ± 45.4

[a] *Monosaccharide composition*: Fuc, fucose; Rha, rhamnose; Ara, arabinose; Gal, galactose; Glc, glucose; Xyl, xylose; Man, mannose; GluA, glucuronic acid; Sul, Sulfate.
* Significantly different at $P \leq 0.05$, number of replicates = 4.

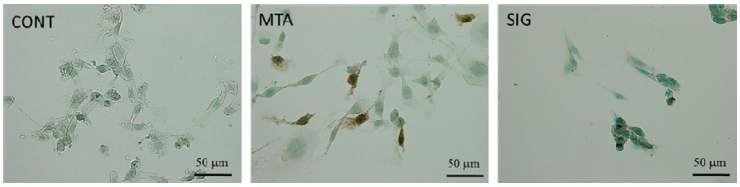

Fig. 2. Images of melanoma B16 cells after exposure to fucoidan: melanoma B16 cells were plated onto cell cultured 2-well permanox slides at a 5×10^4 cells/ml density, and incubated for 48 h in the absence (CONT) and presence of crude fucoidan (MTA, SIG) added at a dosage of 0.8 mg/ml. DAB substrate was used to stain the cells and the images were photographed using an Olympus BX51 microscope following two independent experiments.

0.8 mg/ml MTA and SIG fucoidan resulted in fragmentation and condensation of chromatin, visualized as an intense pyknotic dark brown color within the cell nuclei, as particularly evident for the MTA fucoidan treated cells (Fig. 2). (For interpretation of the references to color in this text, the reader is referred to the web version of the article.)

3.4. Natural killer (NK) cell activity induced by crude fucoidans

The weights of male C57BL/6J mice (Clea Japan Inc., Tokyo, Japan) were recorded and the inherent alertness of the mice was observed daily for 4 days. The records revealed that there were no significant differences in the weights, and that all mice were physically active with no indications of weaknesses during the entire experimental period (data not shown). MTA and SIG fucoidan induced significantly increased levels of splenic NK cell activity in the C57BL/6J mice, assessed as specific lysis after daily *i.p.* injections of the fucoidan samples for four consecutive days (Fig. 3). NK cell activity at 100:1 effector to target ratio increased remarkably in mice treated with fucoidan to $14 \pm 3.8\%$ for MTA and $11 \pm 1.7\%$ for SIG compared to $5.1 \pm 2.1\%$ in the non-treated mice control (Fig. 3). The positive control Poly I:C specific lysis at 100:1 was $26 \pm 9\%$. Statistically significant differences between the effects of MTA and SIG were detected at all effector to target ratios with MTA fucoidan inducing greater NK activity than SIG fucoidan, and the effects of both fucoidans were significantly ($P \leq 0.05$) better than the control (Fig. 3).

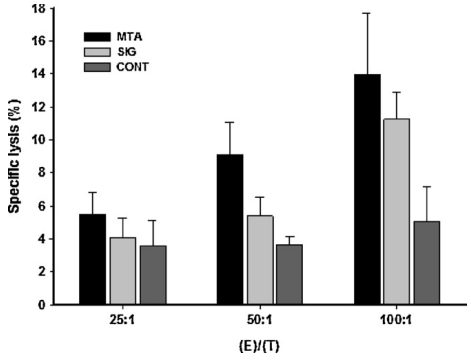

Fig. 3. Natural killer (NK) cell activity in spleen cells of C57BL/6J mice ($n = 3$) against ^{51}Cr-labeled YAC-1 target cells injected *i.p.* for 4 days with either saline or fucoidan (50 mg/kg). Cytotoxicity was determined by measuring ^{51}Cr release at 4 h expressed as percent specific lysis. Positive control Poly I:C specific lysis at 100:1 was $26.2 \pm 8.9\%$. (E)/(T); effector/target. Data are shown as average values $\pm$ s.d.

4. Discussion

The leading cause of cancer deaths worldwide is lung cancer and the incidence of melanoma has risen dramatically over the past few decades [13]. Current therapeutic strategies to combat these cancers provide only minimal benefits and expose cancer patients to significant risks of undesirable side effects and complications. In recent years many studies have focused on the utilization of bioactive compounds derived from biomass of natural origin based on their potential as cancer preventive agents. Fucoidan is a complex sulfated polysaccharide obtained from naturally occurring edible brown seaweeds. For many years, these seaweeds have been a part of Asian diets. A study conducted across the Japanese population investigating the major impact of diet on chronic disease showed that the intake of seaweed was associated with lower mortality from all causes, including lung cancer for men and women [14]. Animal model experiments have shown a direct anti-cancer effect following incorporation of brown seaweeds of the type *Undaria* and *Laminaria* into animal diets with no indications of either weight gain/loss or alterations in the weights of body organs, signifying no direct lethal effect. Although the active components present in these seaweeds have not been determined, it has generally been acknowledged that the effects are most likely attributable to fucoidan [15,16]. Thus, the potential of fucoidan as an outstanding agent for prevention or control of lung and skin cancer is considered promising providing that it does indeed exert cancer-preventive effects.

In this study, we employed crude fucoidan extracted from ground *Sargassum* sp. via a single step extraction using 0.03 M HCl at 90 °C for 4 h, and commercially obtained crude fucoidan from *F. vesiculosus*. We noted that the chemical composition of the seaweed species were distinctively different. In particular, fucose, galactose and glucuronic acid contents differed widely while no differences were noted in the sulfate content (Table 1). Low dosage levels of MTA fucoidan induced more significant reduction of the proliferation of melanoma B16 cells than the SIG fucoidan, but in general the cell proliferation assays revealed almost similar bioactivity effects of MTA and SIG fucoidan on Lewis lung carcinoma and melanoma B16 cells (Fig. 1). These data indicate that the crude fucoidan bioactivity towards these cell lines was probably related to the sulfate groups present in the fucoidan structure rather than the presence of significant amounts of fucose. The same observation was reported by Yamamoto et al. [17] with respect to the influence of fucose content of fucoidan on the growth inhibition of Sarcoma-180 cells; Yamamoto et al. [17] thus found that sulfation rather than fucose content of crude fucoidan elicited anti-tumor activity.

The sulfate contents of MTA and SIG fucoidan were 38.4% and 34.2%, respectively (Table 1). These results agreed with those of Koyanagi et al. [18] who reported that purified fucoidan from *F. vesiculosus* containing 32.6% sulfate was a potent anti-angiogenic agent and that oversulfation of fucoidan might have intensified the anti-angiogenesis and anti-tumor activity of fucoidan. Addi-

tionally, the anti-coagulant activity of sulfated polysaccharides has been reported to be related to the sulfation level [19]. In contrast, Cumashi et al. [20] studied nine different modified fucoidans and found that neither the content of fucose, sulfate nor other structural features of the polysaccharide backbone significantly affected the efficacy of the fucoidan bio-activity. These conflicting conclusions about the bio-activity of fucoidan have paved the way for further exploration of this topic. While the present study is too premature to elucidate the relationship between the composition of crude fucoidan and its biological activities, it has shown that crude fucoidan exerts significant anti-proliferative effects on both lung and skin cancer cells.

In the past, most anti-cancer studies of fucoidan have used purified fractions, and showed inhibitory effects relating to metastasis [20], angiogenesis [18] and suppression of growth in a variety of cancer cells [21,22]. Nonetheless, the ability of crude fucoidan to inhibit cancer cells has also been documented previously based on various *in vivo* and *in vitro* studies, including growth inhibition of implanted Sarcoma-180 cells [17] and induced apoptosis of HCT-15 colon carcinoma cells [23]. Still, our knowledge of the effect of crude fucoidan on lung and skin carcinogenesis is limited; therefore, detailed investigations must be carried out to elucidate the underlying mechanisms behind the effects. In the present work, we demonstrated that crude fucoidan effectively inhibits Lewis lung carcinoma and melanoma B16 cells at concentrations between 0.1 and 1.0 mg/ml (Fig. 1). We also examined whether the observed tumor inhibition by crude fucoidan was due to anti-tumor activity or direct cytotoxicity. Overall, the *in vitro* cytotoxic activity of fucoidan against Lewis lung carcinoma and melanoma B16 cells increased in a dose-dependent fashion (Fig. 1). As such, it may be concluded that crude fucoidan exhibits a weak, direct cytotoxic effect against these carcinogenic cells. Furthermore, the absence of weight loss and frailness in the mice treated *i.p.* with MTA and SIG fucoidan (data not shown) suggest that inhibition of LLC and MC cell growth was attributed, at least in part, to its anti-tumor activity.

Crude fucoidan and some seaweed extracts have immunomodulating activity, such as NK cell activity which enhances anti-tumor activities [9,24]. NK cells appear to represent a first line of defense against the metastatic spread of blood-borne tumor cells. Normal NK cell activity may also be important in immune surveillance against tumors [25]. We studied NK cell activity in the presence and absence of crude MTA and SIG fucoidan in male C57BL/6J mice. The cytolytic activation of NK cells against the YAC-1 target cells was significantly improved in the splenocytes of mice after 4 days of *i.p.* treatments with crude fucoidan (Fig. 3). Crude fucoidan thus exerted anti-tumor activity through an enhancement of the immune-response. It was previously shown that fucoidan from *Undaria pinnatifida* enhances immune response and acts as an immunopotentiator in tumor-bearing mice, leading to anti-tumor effectiveness [16]. NK cells produce a number of cytokines, such as interferon-γ (IFN-γ) production by T cells which have been stimulated by interleukin-12 (IL-12) [26]. It has been reported that stimulation by IL-12 alone produces only moderate augmentation of NK cell cytotoxicity. However, IL-12 increases the catalytic activity of lymphocytes against autologous targets when in synergy with interleukin-2 (IL-2) [27]. Hence, stimulation with IL-2 and IL-12 promotes the secretion of IFN-γ; the mechanism behind fucoidan enhanced NK cell activation may be similar (Fig. 3).

Previous studies have shown that fucoidan induces apoptosis in HT-29 colon cancer cells [7], MCF-7 human breast cancer cells [28], and HS-Sultan human lymphoma cells [6]. In this study, crude fucoidan exerted activity through inhibition of growth of Lewis lung carcinoma and melanoma B16 cells. As such, we studied whether crude fucoidan exerted the activity through inducing apoptosis. Histochemical staining with the DAB substrate showed morphological changes of the melanoma B16 cells, as indicated by an intense dark brown color within the cell nuclei (Fig. 2). A distinct morphological change of cells by apoptosis includes modification of the cytoskeleton which results in membrane bleeding, condensation of chromatin and degradation of the DNA into fragments [26]. In the present work crude fucoidan was shown to induce apoptosis; previous reports have suggested that fucoidan induces apoptosis via the activation of caspase-3 in human HS-Sultan cells [6], via caspase-8 dependent pathways in MCF-7 cells [28] and through the activation of caspases via both death receptor-mediated and mitochondria-mediated apoptotic pathways [7]. The detailed mechanisms of the activation pathways clearly deserve further investigation.

5. Conclusions

We have examined the bioactivity of crude fucoidan through evaluation of its efficacy in controlling or inhibiting lung and skin cancer cell proliferation *in vitro*. The bioactivity of crude fucoidan towards these two types of cell lines was probably generated by the sulfate groups in the fucoidan structure. These findings need to be examined further to elucidate the underlying factors of fucoidan bioactivity. The study showed that crude fucoidan induces apoptosis of melanoma B16 cells and exerts anti-tumor activity through inhibition of the growth of Lewis lung carcinoma and melanoma B16 cells. In the present work, NK cells of mice treated with crude fucoidan acted as the principal effectors mediating tumor cell death. Overall, anti-tumor activity promoted by crude fucoidan was based on the enhancement of NK cell activity. Crude fucoidan from *Sargassum* sp. and *F. vesiculosus* thus appears to be a potent lung and skin cancer-preventive agent and its mode of action is associated with the immune response.

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Article

Fucose-Containing Sulfated Polysaccharides from Brown Seaweeds Inhibit Proliferation of Melanoma Cells and Induce Apoptosis by Activation of Caspase-3 *in Vitro*

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Abstract: Fucose-containing sulfated polysaccharides (FCSPs) extracted from seaweeds, especially brown macro-algae, are known to possess essential bioactive properties, notably growth inhibitory effects on tumor cells. In this work, we conducted a series of *in vitro* studies to examine the influence of FCSPs products from *Sargassum henslowianum* C. Agardh (FSAR) and *Fucus vesiculosus* (FVES), respectively, on proliferation of melanoma B16 cells and to investigate the underlying apoptosis promoting mechanisms. Cell viability analysis showed that both FCSPs products, *i.e.*, FSAR and FVES, decreased the proliferation of the melanoma cells in a dose-response fashion, with FSAR being more potent at lower dosages, and FVES being relatively more anti-proliferative than FSAR at higher dosages. Flow cytometric analysis by Annexin V staining of the melanoma cells exposed to the FCSPs products confirmed that both FSAR and FVES induced apoptosis. The FCSPs-induced apoptosis was evidenced by loss of plasma membrane asymmetry and translocation of the cell membrane phospholipids and was accompanied by the activation of caspase-3. The FCSPs bioactivity is proposed to be attributable to distinct structural features of the FCSPs, particularly the presence of sulfated galactofucans (notably in

S. henslowianum) and sulfated fucans (notably in *F. vesiculosus*). This study thus indicates that unfractionated FCSPs may exert bioactive effects on skin cancer cells via induction of apoptosis through cascades of reactions that involve activation of caspase-3.

Keywords: fucoidan; anti-tumor; sulfated polysaccharides; bio-activity; apoptosis; fucose

1. Introduction

Fucose-containing sulfated polysaccharides (FCSPs) designate a group of diverse polysaccharides that can be extracted from brown seaweeds of the class Phaeophyceae. This seaweed class includes the order Fucales, in which seaweed species such as *Fucus* sp. and *Sargassum* sp. belong. The most studied FCSPs, originally called fucoidin, fucoidan or just fucans, have a backbone built of (1→3)-linked α-L-fucopyranosyl residues or of alternating (1→3)- and (1→4)-linked α-L-fucopyranosyl residues [1,2]. These fucopyranosyl residues may be substituted with short fucoside side chains or sulfate groups at C-2 or C-4, and may also carry other minor substitutions, e.g., acetate, xylose, mannose, glucuronic acid, galactose, or glucose [3–5]. Brown seaweed FCSPs also include sulfated galactofucans with backbones built of (1→6)-β-D-galacto- and/or (1→2)-β-D-mannopyranosyl units. In addition to sulfate these backbone residues may be substituted with fucosides, single fucose substitutions, and/or glucuronic acid, xylose or glucose substitutions [4]. Recently it has been understood that the compositional and structural features of FCSPs differ significantly among seaweed species and that these features are markedly influenced by the conditions used to extract them [3,6].

FCSPs of different degrees of purity and composition, extracted from brown seaweeds such as *Sargassum* sp. and *Fucus* sp., have been documented to have a wide range of biological activities including anticoagulant [7,8], antithrombotic [8], anti-inflammatory [9], anti-viral [10,11]; and notably anti-tumoral effects [8,12,13]. Unfractionated FCSPs have thus specifically been found to reduce cell proliferation of lung carcinoma and melanoma cells *in vitro*; to exert immunopotentiating effects in tumor bearing animals; and to activate natural killer cells in mice leading to increased anti-tumor effectiveness [13–16]. Kim *et al.* [17] applied a crude polysaccharide composed predominantly of sulfated fucose from *Fucus vesiculosus* to human colon cancer cells *in vitro*, and concluded that this crude brown seaweed polysaccharide extract can induce apoptosis, and provided data that suggested that the apoptosis was induced via activation of caspases. Moreover, commercially available crude FCSPs ("fucoidan") extracted from *F. vesiculosus* have been reported to inhibit proliferation and induce apoptosis on human lymphoma HS-Sultan cells lines by activation of caspase-3 [18]. Recently, we have reported that crude FCSPs extracted from a *Sargassum* sp. and from *F. vesiculosus*, respectively, induce growth inhibition and apoptosis of melanoma B16 cells *in vitro* [13]. When injected intraperitoneally into mice over four days, these same unfractionated FCSPs were found to induce enhanced natural killer cells (NK cells) activity to result in specific lysis of YAC-1 cells (a murine T-lymphoma cell line sensitive to NK cells) [13]. Previous reports with human HS-Sultan cells and MCF-7 cells, respectively, have suggested that the FCSPs induced apoptosis initiation may take place via activation of caspase-3 and caspase-8 dependent pathways, respectively [18,19], but no firm evidence has been established regarding the exact mechanism responsible for the apoptotic action

of the FCSPs. The objective of the present study was, therefore, to examine whether the anti-proliferative action and apoptosis of melanoma B16 cells induced by FCSPs derived from *Sargassum henslowianum* C. Agardh and *Fucus vesiculosus*, are accompanied by increased caspase-3 activity. We also wanted to evaluate whether any structural features of the FCSPs might be crucial for bioactivity. In this study, we present the different structural features of the FCSPs derived from *S. henslowianum* and *F. vesiculosus* as assessed by IR and ^{1}H NMR spectroscopy and show that these FCSPs exert bioactive effects that inhibit the proliferation of melanoma B16 cells by apoptosis. We also show that the antiproliferative effects and the apoptosis are accompanied by activation of caspase-3.

2. Results

2.1. FCSPs Chemical Composition

The compositional analysis of the fucose-containing sulfated polysaccharide products from *S. henslowianum* C. Agardh (FSAR) and *F. vesiculosus* (FVES), respectively, showed that the FSAR product was mainly made up of uronic acid and fucose, with a significant level of sulfate, and minor amounts of other monosaccharides, mainly galactose and mannose (Table 1). The FVES product had a similar monosaccharide profile and a similar sulfation level, but the amounts of fucose, galactose and xylose were significantly higher than in FSAR; whereas the uronic acid and mannose levels were lower (Table 1).

Table 1. Monosaccharide composition and sulfate content of the fucose-containing sulfated polysaccharides: *Sargassum henslowianum* C. Agardh (FSAR) derived from *S. henslowianum* C. Agardh and *Fucus vesiculosus* (FVES) derived from *F. vesiculosus*, respectively.

Samples	Monosaccharide Composition * in mg/g DW								
	Fuc **	**Rha**	**Ara**	**Gal ****	**Glc**	**Xyl ****	**Man ****	**UA ****	**Sulfate**
FSAR	31 ± 2	1.6 ± 0.1	0.2 ± 0.1	14 ± 1	4.2 ± 0.1	4.2 ± 0.3	5.7 ± 0.5	123 ± 7	384 ± 26
FVES	139 ± 5	2.0 ± 0.6	2.8 ± 0.2	28 ± 1	2.5 ± 1.8	13 ± 2	0.2 ± 0.4	19 ± 2	342 ± 45

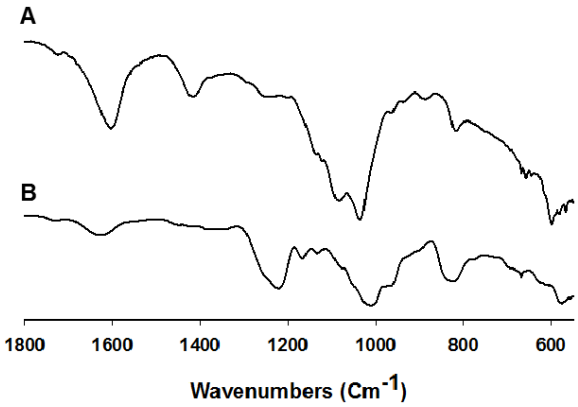
***** Monosaccharide composition: Fuc = fucose, Rha = rhamnose, Ara = arabinose, Gal = galactose, Glc = glucose, Xyl = xylose, Man = mannose, UA = uronic acid; ****** Significantly different levels among FSAR and FVES at $P \leq 0.05$, number of replicates = 4.

2.2. IR and ^{1}H NMR Spectra of FCSPs

The FCSPs were analyzed to determine if their infrared absorption properties were similar to the previously reported fucoidan IR absorption data [2,20]. The spectra of the FSAR and FVES samples scanned between wavenumbers 4000 and 400 cm^{-1} both exhibited major absorption bands at around 3340 and 3420 cm^{-1} that were interpreted as being due to O–H stretching (data not shown). The IR spectra between 1800 and 500 cm^{-1} (Figure 1a,b) revealed small but distinct bands for both the samples at 1720 cm^{-1} which indicated the presence of *O*-acetyl groups [21], whereas the absorption bands at ~1610 to 1620 cm^{-1} (Figure 1a,b), most pronounced for the FSAR sample, indicated uronic acid [20]. The FSAR sample showed an intense IR band at around 1400–1470 cm^{-1} which could be attributable to scissoring vibration of CH_2 (galactose, mannose) and asymmetric bending vibration of CH_3 (fucose, *O*-acetyls) as suggested previously for absorption at around 1455 cm^{-1} by Synytsya *et al.* [22]. The

absorption band at 1240 cm^{-1} observed for both samples, but being particularly prevalent for the FVES sample, was assigned as S=O stretching vibration, indicating the presence of esterified sulfate [20]. A similar absorption pattern around 820–840 cm^{-1} was observed for both FCSPs: The FSAR infrared spectrum showed an absorption band at 817 cm^{-1} (Figure 1a) whereas the FVES infrared spectrum displayed a broader absorption band at 838 cm^{-1} and a small shoulder of absorption at 822 cm^{-1} (Figure 1b). Since IR adsorption at 840 cm^{-1} has been reported to be due to sulfate groups at the axial C-4 position whereas sulfate groups at the equatorial C-2 and/or C-3 positions have been reported to give a small absorption at 820 cm^{-1} [2], the observed absorption bands at 820–840 cm^{-1} were interpreted as being indicative of sulfate groups.

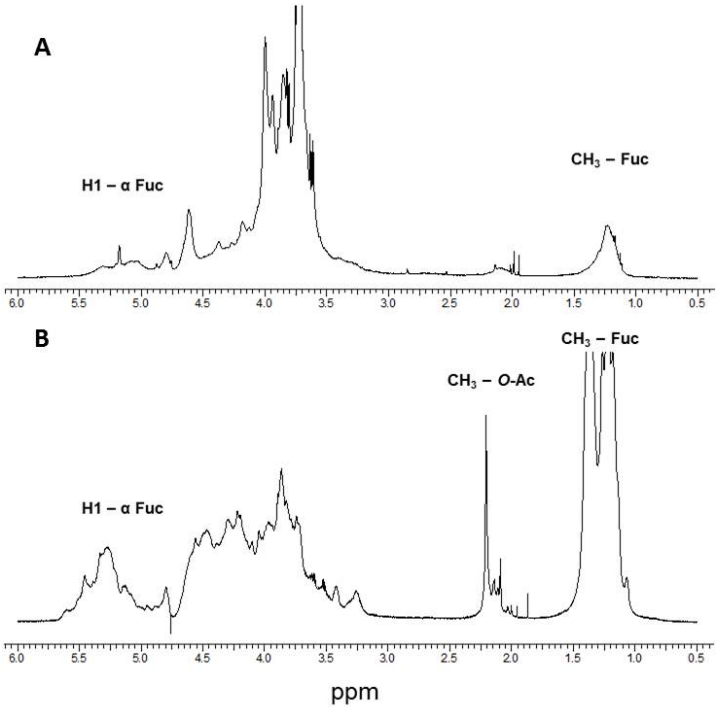
Figure 1. Infrared analysis of fucose-containing sulfated polysaccharides (FCSPs) from (**a**) *Sargassum henslowianum* C. Agardh (FSAR) and (**b**) *Fucus vesiculosus* (FVES) scanned between 1800 and 550 cm^{-1}.



The proton NMR spectra (^2H_2O) of the FSAR and the FVES samples were complex with broad signals and with several signals in the chemical shift of the envelope of anomeric signals at 5.0–5.5 ppm (Figure 2a,b). The presence of the signals at 5.0–5.5 ppm is consistent with the presence of α-L-fucopyranosyl [23]. The ^{1}H-NMR spectra also both contained peaks at 1.1–1.3 ppm, with the signals for the FVES sample being particularly strong (Figure 2a,b). Previously, such high-field region signals at 1.1–1.3 ppm have been assigned to a C6 methyl proton group of L-fucopyranose [22] whereas several intense and narrow signals at 2.14–2.21 ppm have been attributed to CH_3 protons of *O*-acetyl groups [5]. The narrow and intense signals at 5.10 and 5.18 ppm in the chemical shift of the envelope of the anomeric proton of FSAR (Figure 2a) were reported earlier and assigned to α3-linked and α3,4-linked L-fucopyranose residues for fucoidan from *Hizikia fusiformis* a.k.a *Sargassum fusiformis* [24]. The high field region signals 1.24 and 1.20 ppm (Figure 2a) were assigned to α3-linked 2-mono-*O*-sulfated and α3-linked unsulfated L-fucopyranose residues [21]. Moreover the intense signals at 4.37 and 3.99 ppm (Figure 2a) were assigned to the presence of 4-linked 2-mono-*O*-sulfated L-fucopyranose residues [21]. The independent signal, 4.61 ppm (Figure 2a) was assigned to a 3-linked D-galactopyranosyl residue when compared with the data of Farias *et al.* [25]. The

FVES had an intense signal at 5.45 ppm (Figure 2b), which was assigned to α3-linked 2-mono-*O*-sulfated L-fucopyranose residues, whereas the signals at 5.40, 4.58 and 4.39 ppm (Figure 2b) were assigned to be due to di-sulfated residues, *i.e.*, α3-linked 2,4-di-*O*-sulfated L-fucopyranose residues [26]. Hence in general, the ^{1}H NMR confirmed the anticipated FCSPs structures of the two samples.

Figure 2. One-dimensional ^{1}H NMR spectra of crude FCSPs from (**a**) *Sargassum henslowianum* C. Agardh (FSAR) and (**b**) *Fucus vesiculosus* (FVES) in D_2O obtained using an INOVA 600 NMR spectrometer (Agilent Technologies, Tokyo, Japan).

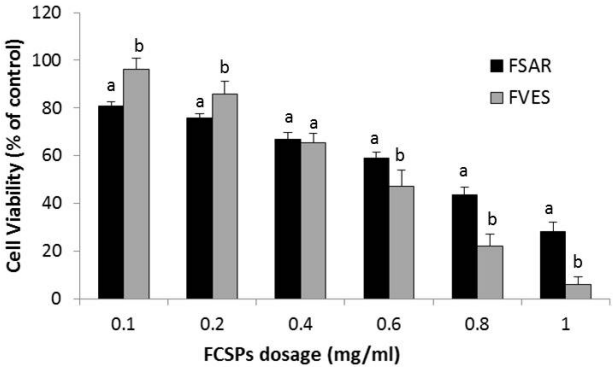


2.3. Anti-Proliferative Effects of the FCSPs

The viability of melanoma B16 cells treated with the FSAR and FVES products, respectively, was determined via measurement of cell proliferation using an MTT based colorimetric assay. Both FCSPs products (FSAR and FVES) decreased the viability of melanoma B16 cells in a dose-dependent fashion, after 24 h of incubation of 6×10^4 cells density per well (Figure 3). In particular, a pronounced cell viability reduction was noticed after the addition of low levels, 0.1 mg/mL, of FSAR, producing a cell viability of ~80% of the control, and cell proliferation was halted gradually as the FCSPs dosage level increased (Figure 3) indicating moderate cytotoxicity. The FVES treated cells showed the same trend, but the FVES product generally induced a lower anti-proliferative effect than the FSAR product at the lower FCSP addition levels ($P \leq 0.05$), but a significantly higher effect than FSAR ($P \leq 0.05$) at the higher addition level, producing a drastic reduction of the proliferation of melanoma B16 cells leaving only ~6% of the cells viable at an FCSPs addition level of 1 mg/mL (Figure 3). The viability

reduction pattern induced by the two FCSPs on the melanoma cells were in complete accord with previously published data [13].

Figure 3. MTT based colorimetric assay of cell viability of melonama B16 cells after treatment for 24 h with different dosage levels of crude fucose-containing sulfated polysaccharides from *Sargassum henslowianum* C. Agardh (FSAR) and *Fucus vesiculosus* (FVES), respectively. Cell density was 6×10^4 cells per well. [a,b] indicate statistically significantly different ($P \leq 0.05$) cell viability levels after treatment with the two FCSP products at the same dosage level (mg/mL) ($n = 4$).

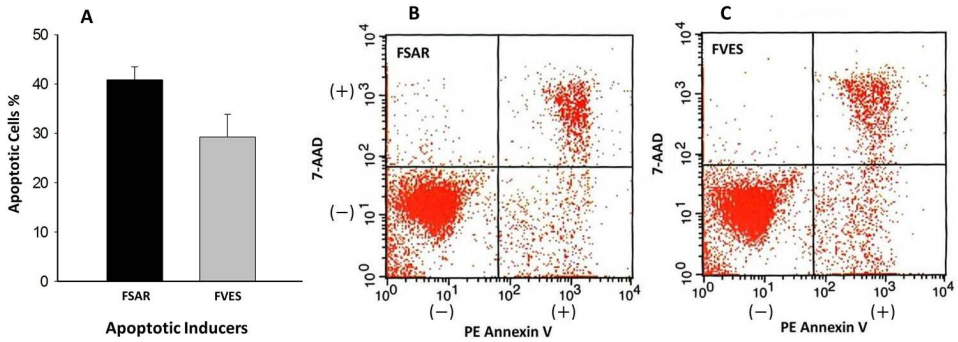


2.4. Apoptosis of Melanoma Cells by FCSPs

Programmed cell death or apoptosis is characterized by certain morphological cell changes such as loss of plasma membrane integrity in addition to internucleosomal DNA cleavage. One of the earliest apparent changes in cells undergoing apoptosis is the translocation of the cell membrane phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane. This change in the cell membrane is now recognized as an early, essential feature of apoptosis. The translocation exposes the phosphatidylserine to the external cellular environment and this is a feature which can be measured by exposing the cells to fluorochrome-conjugated phospholipid-binding proteins such as phycoerythin (PE)-labelled Annexin V (PE-Annexin V). Such staining with Annexin V is typically used in conjunction with a vital dye such as 7-amino-acticomycin (7-AAD) to identify early stages of apoptotic cells (Annexin V^+, 7-AAD^-) which accompany the later apoptosis stages (both Annexin V and 7-AAD are positive). Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells undergoing apoptosis are permeable to 7-AAD. Figure 4 shows the number of melanoma cells undergoing apoptosis (% relative to a control not exposed to FCSPs) and the flow cytometric scan data of Annexin V staining induced by exposure of the melanoma cells to 0.2 mg/mL of the seaweed FCSPs samples from *S. henslowianum* (FSAR) and *F. vesiculosus* (FVES), respectively. Both FCSPs products induced significant apoptosis of the melanoma cells: The FSAR product appeared to induce a more potent apoptotic effect than the FVES product (Figure 4a) since the relative number of melanoma cells undergoing apoptosis (% relative to a control sample not exposed to FCSPs) induced by the FSAR sample was significantly higher (41 ± 3%) than the apoptotic effect of

the FVES exposure (30 ± 5%). The data corresponded to the fluorescence-activated cell sorting (FACS) scan showing the accumulation of intense dots-color in cells that underwent the latest apoptosis stage (Figure 4b,c: both Annexin V and 7-AAD positive). The FVES sample induced more early apoptosis (Figure 4c than the FSAR, as characterized by the build-up of disperse dots-color (Annexin V^+ and 7-AAD^-) indicating loss of plasma membrane asymmetry. The data were in accordance with the anti-proliferative effects of the FCSPs treatments (Figure 3).

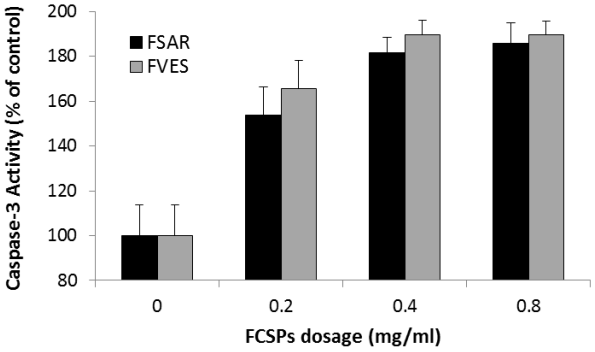
Figure 4. Flow cytometric analysis by Annexin V staining of Melanoma B16 cells treated for 24 h with 0.2 mg/mL crude fucose-containing sulfated polysaccharide (FCSP) products extracted from *S. henslowianum* C. Agardh (FSAR) and *F. vesiculosus* (FVES), respectively. (**a**) Apoptosis induced by FSAR (41 ± 3%, n = 2), FVES (30 ± 5%, n = 2); control 11.66 % (data not shown) (**b**) FSAR data and (**c**) FVES data for FACS scans of FCSP treated Melanoma 16 cells that were viable and not undergoing apoptosis (Annexin V^- and 7-AAD^-); undergoing early apoptosis, with membrane integrity intact (Annexin V^+ and 7-AAD^-); in the latest stage apoptosis and dead (Annexin V^+ and 7-AAD^+), respectively.



2.5. FCSPs Activation of Caspase-3

In general, activation of caspase-3 initiates apoptosis in mammalian cells. The caspase-3 colorimetric assay employed in the present study is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage of the pNA-labeled substrate DEVD-pNA. The activity of caspase-3 was augmented after treatment of the melanoma cells for 24 h with the FCSPs from *S. henslowianum* (FSAR) and *F. vesiculosus* (FVES) (Figure 5). The recorded caspase-3 activity increased significantly in a dose-dependent manner in response to the FCSPs treatment dosage (0–0.8 mg/mL), *i.e.*, from 100% of control at 0, to ~180% of the control response at 0.8 mg/mL ($P \leq 0.05$) (Figure 5). No significant differences in the responses induced by the two types of FCSPs were recorded within the individual dosages of the FSAR and FVES treatments (Figure 5). The same trend of caspase-3 activity was observed in a 48 h treatment of melanoma cells with the FSAR and FVES samples (data not shown).

Figure 5. Activation of caspase-3 after treatment of melanoma B16 cells with different dosages of FCSPs from *S. henslowianum* (FSAR) and *F. vesiculosus* (FVES). For each dosage treatment the caspase-3 activity was assayed on a cytosolic extract of melanoma B16 cells with a DEVD-pNA substrate (contact at 37 °C for 1 h) and spectrophotometric detection by measuring the absorbance at 405 nm (n = 2).



3. Discussion

The incidence of melanoma skin cancer has risen dramatically over the past few decades [27]. Because of the significant risk and undesirable effects of known cancer therapeutic strategies, many studies have evaluated the possible protective effects of bioactive compounds of natural origin. Fucose-containing sulfated polysaccharides (FCSPs) derived from naturally grown brown seaweeds by aqueous extraction have been shown to exert potentially beneficial bioactivities, including immuno-modulatory, anti-inflammatory and anti-tumorigenic effects. In keeping its natural properties, FCSPs must be extracted from brown seaweeds by use of a mild processing treatment and a minimal number of extraction steps.

Brown seaweeds constitute a part of the conventional diet in several Asian countries, especially in Japan, and in a Japanese cohort study the intake of seaweeds has been associated with lower mortality from all chronic diseases including cancer [28]. It has recently been demonstrated that FCSPs from brown seaweeds exert growth inhibitory activity on certain cancer cell lines *in vitro* [13,17,18]. Incorporation of brown seaweeds into animal diets has also revealed cancer inhibitory effects with no direct lethal consequences [29,30]. Natural FCSPs from brown seaweeds may therefore have significant potential as protective agents to control or prevent skin cancer provided that the FCSPs do indeed exert cancer-preventive effects.

In this study, in accordance with previous data [13], we found that unfractionated FCSPs, *i.e.*, FSAR and FVES, extracted from the brown seaweeds *S. henslowianum* and *F. vesiculosus,* respectively, were composed of fucose, galactose, xylose, mannose and glucuronic acid, and showed that the fucose, galactose and glucuronic acid contents differed significantly among the two FCSPs products, but that their sulfate contents were similar (Table 1).

We also found both distinct differences and several similarities in the structural make-up of these FCPSs by use of FTIR and ^{1}H NMR spectroscopy. The FT-IR analyses thus corroborated the presence

of sulfate groups in both the FSAR and the FVES sample (Figure 1). The IR spectra indicated that the sulfate substitutions of the FCSPs extracted from the *Sargassum* sp. (FSAR) were located in the equatorial C-2 and/or C-3 positions as depicted by absorption bands at 817 cm^{-1}. This finding was in agreement with data reported for fucoidan fractions isolated from *Sargassum stenophyllum* [4]. However, Duarte *et al.* [4] also reported that two other saccharide fractions from *S. stenophyllum* had an absorption band at 837 cm^{-1} indicating sulfate groups at the C-4 positions of the structural monosaccharides [4]. The spectra of the FCSPs from *F. vesiculosus* (FVES) displayed an absorption band at 838 cm^{-1} with a small shoulder at ~822 cm^{-1} indicating sulfate groups at both the C-4 and the C-2 position (Figure 1). This finding corresponds to previously reported ^{1}H NMR data of FCSPs from *F. vesiculosus* that have indicated a typical structure of algal fucoidan consisting of α3-linked 2-mono-*O*-sulfated L-fucopyranose residues, and/or α3-linked 2,4-di-*O*-sulfated L-fucopyranose residues [2,26]. Small disparities in the IR spectra from different published reports can be due to factors such as sample handling and the FCSPs extraction procedure employed.

The present study also aimed at establishing whether crude FCSPs extracted from *Sargassum henslowianum* C. Agardh (FSAR) contained fucoidan-like structures composed of α-3-linked or/and α-3,4-linked L-fucopyranose residues. Even though signals consistent with the presence of α-L-fucopyranose entities were recorded (with ^{1}H NMR signals at 5.10 and 5.18 ppm, Figure 2a), the probability that the FSAR may contain a cocktail of polysaccharides is likely. Hence, the ^{1}H NMR spectra also showed that the FSAR sample contained 3-linked D-galactopyranose residues as indicated by an independent signal at 4.61 ppm (Figure 2a). β-(1→)3-linked galactopyranose residues are known to be a typical structural feature of seaweed polysaccharides, from e.g., *Laminaria angustata* var. *longissima*, *Botryocladia occidentalis* [25,31]. However, another possibility might be that the FSAR sample was not composed of a mixture of different types of polysaccharides but rather, that the sample consisted of one type of a highly complex hetero-polysaccharide as suggested by Duarte *et al.* [4] for the fucoidans from *Sargassum stenophyllum.* It can safely be said that the ^{1}H NMR spectra of the FCSPs samples were complex and overlapping. It is therefore difficult to draw any definite conclusions about the detailed structural features and differences among the two FCSPs; the detailed elucidations of the definite structural details were also beyond the scope of this present study, but clearly deserve further investigation. Nonetheless, the data confirmed that the diversity, *i.e.*, the compositional and structural complexity of (potentially bioactive) algal fucose-containing sulfated polysaccharides, is much wider than originally believed.

The biological activities of the FCSPS against skin cancer cells were investigated *in vitro*, and the results revealed that both FSAR and FVES can exert anti-proliferative effects on melanoma B16 cells *in vitro*. The FSAR sample induced more significant reductions of the cell viability of melanoma cells than the FVES sample at low dosage levels (Figure 3). At higher dosages, the FSAR treatment still induced gradually more loss of cell viability, but the FVES had more potent anti-proliferative effects at higher dosages than FSAR which could indicate direct cell toxicity. The bioactivities of these FCSPs may be attributable to their distinct structural features, notably the level of sulfation (charge density), the distribution (e.g., random *versus* clustered) and bonding of the sulfate substitutions, as well as other specific structural features of the sulfated fucans and the sulfated galactofucan complexes. The sulfate groups of FVES were substituted at the C-2 and C-4 position of the fucose substituents, typical for fucoidan from *F. vesiculosus*, and consistent with previously published data indicating that the sulfate

groups were substituted at C-2 of α3-linked L-fucopyranose residues in fucoidan from e.g., *Fucus evanescens* [21]. In contrast, the sulfate substitutions in the FSAR were interpreted to be mainly at the C-2 and/or C-3 positions of the monosaccharides according to the IR spectra (Figure 1); the observation of C-2 linked sulfate groups agreed with the data mentioned above for fucoidan from *Fucus* spp. [3,21], but is also in agreement with the proposition that the sulfate groups were substituted at C-2 on the 3-linked galactopyranose residues [25]. The possible presence of sulfated, 3-linked galactan in the structure of FSAR may contribute to the efficacy of FSAR to induce anti-proliferative effects as it has been reported that 2-*O*-sulfated 3-linked galactan is more bioactive than 2-*O*-sulfated 3-linked fucans and 3-*O*-sulfated 4-linked galactan [32–34].

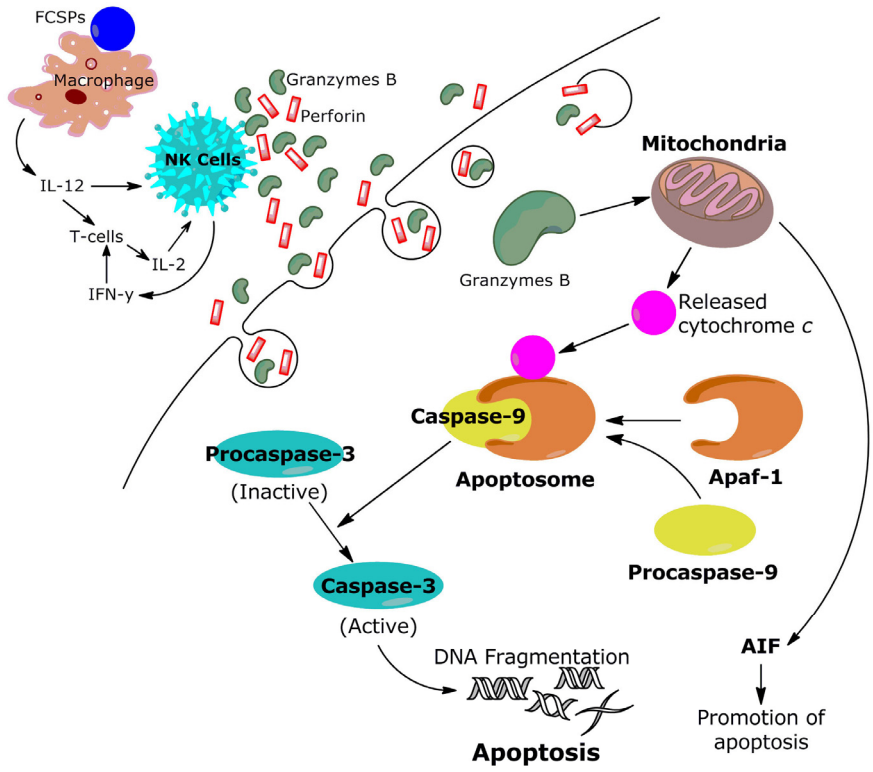
The findings that the FCSPs induced apoptosis of the melanoma B16 cells *in vitro* were in agreement with recent reports [13,16,35], but the differential apoptotic efficacies, and the dose-response effects of differently structured FCSPs (Figure 4) have not been reported earlier. In particular, it is a novel finding that significantly different sulfated, polysaccharide structures from brown seaweeds—as evaluated in the present work—exert relatively similar apoptotic effects on melanoma cells. The results of this work thus indicate that not only the well-studied, classical type of FCSPs having a backbone made up of (1→3)-linked α-L-fucopyranosyl or of alternating (1→3)- and (1→4)-linked α-L-fucopyranosyl residues have potential tumor-preventing effecs, but also that the more complex sulfated fucose-rich galacto-mannans from *Sargassum* spp. exert promising cancer-preventive effects. The principal objective of this study was to assess whether any structural features of the FCSPs might be crucial for bioactivity, and the data suggest that the sulfate substitutions, and not necessarily only the fucose-backbone structure itself, confer this decisive bioactivity. It is however important to investigate whether other differently structured FCSPs may exert similar growth inhibitory and apoptosis inducing effects on cancer cells.

In this work we noted that both FCSPs activate caspase-3 in a dosage-response fashion (Figure 5). These findings affirmed the results reported previously which have shown that FCSPs ("fucoidan") from *F. vesiculosus* induce apoptosis in human lymphoma HS-Sultan cell lines and in HT-29 and HCT116 human colon cancer cells *in vitro*, and moreover that the exposure of these cells to the *F. vesiculosus* FCSPs appear to activate caspase-3 [17,18]. The *F. vesiculosus* FCSPs treatment was also shown to enhance mitochondrial membrane permeability of human colon cancer cells *in vitro*, and to induce cytochrome c and Smac/Diablo release from the mitochondria [17]. It has also been reported that pretreatment of HT-29 and HCT116 colon cancer cells with individual caspase-8 or caspase-9 inhibitors (Z-IETD-FMK and Z-LEHD-FMK, respectively) prior to fucoidan exposure reduced the levels of caspases, including caspase-3 [17]. It has likewise been shown that pretreatment of human lymphoma HS Sultan cells with a pan-caspase inhibitor, z-VAD-FMK, reduced fucoidan-induced apoptosis [18]. Hence, the available data support the proposition that fucoidan-induced apoptosis occurs through caspase activation pathways. The cascade mechanism by which the caspase-activation is presumed to take place via the mitochondria-mediated apoptotic pathway is illustrated in Figure 6.

Loss of plasma membrane is one of the earliest features of apoptosis and Annexin V staining can identify apoptosis at an early stage. However this assay does not distinguish between cells that have undergone apoptotic death *versus* those that have died as a result of a necrotic pathway, because in either case the dead cells will stain with both Annexin V and 7-AAD. Both early and later apoptosis stages were observed by the FACS scanning indicating that the FCSPs had a direct apoptotic effect on

the melanoma B16 cells (*in vitro*) (Figure 4). The direct apoptotic action of the FCSPs was probably due to the interaction of the highly negative charge density of the FCSPs with the melanoma B16 cells (as a result of the sulfation). Recently, we reported that crude fucoidan from *Sargassum* sp. could trigger apoptosis indirectly by enhancing the activity of natural killer (NK) cells activity *in vivo* [13]. NK cells produce immunologically important cytokines, notably IFN-γ, which can promote the activation of T-cells to produce interleukin-2 and -12 that in turn further enhance the NK cell activation [14,36].

Figure 6. Proposed mechanism for inhibition of the proliferation of melanoma cells by FCSPs: Activation of macrophages via membrane receptors, which leads to the production of cytokines that enhance NK cell activation. Activated NK cells release Granzyme B and perforin through granule exocytosis into the space between NK cells and melanoma cells to initiate caspase cascades in melanoma cells. Assimilation of Granzyme B by the tumor cells is facilitated by perforin. Granzyme B then initiates apoptosis by triggering the release of mitochondrial cytochrome c and apoptosome formation leading to caspase-3 activation, which in turn translocates the nucleus causing DNA fragmentation—the distinct morphological change of cells by apoptosis [36,37].



The apoptosis induced by FCSPS via the activation of caspase-3 was reported previously to be mediated through a mitochondrial pathway [17–19,38]. However, it remains to be determined whether

differences in FCSPs structures will influence the apoptotic mechanism, including the mitochondrial pathway apoptosis cascade. The route of the mitochondrially dependent apoptotic pathway is the release of apoptosis-inducing factor (AIF) and cytochrome *c* from the inner mitochondrial membrane into the cytosol. Cytochrome *c* interacts with Apaf-1 (apoptotic protease activating factor 1) and procaspase-9 to form the active apoptosome. The apoptosome then initiates the cleavage of procaspase-3, producing active caspase-3, which initiates the execution phase of apoptosis by proteolysis of substances whose cleavage commits the cell to apoptosis [39] (Figure 6). The influence of the different FCSPs structures on the mitochondrial membrane permeability and electric potential requires further study. We hope in the future to investigate the bioactivity and mechanism of FCSPs on certain degenerative diseases *in vivo* and to further elucidate specific molecular targets of FSCPs for inhibition of cancer cells.

4. Experimental Section

4.1. Chemicals

Dried *S. henslowianum* C. Agardh was obtained from Viet Delta Ltd. (Ho Chi Menh, Vietnam) and the Fucose-containing sulfated polysaccharides (FCSPs) from the *S. henslowianum* (FSAR) were extracted in our laboratory (see below). Crude fucoidan from *F. vesiculosus* (FVES) was obtained from Sigma-Aldrich (Steinheim, Germany); according to the product description the FVES had been prepared from *F. vesiculosus* via the extraction method described by Black and Dewar [40]. Hydrochloric acid (37%), D-glucose and D-xylose were purchased from Merck (Darmstadt, Germany). Trifluoracetic acid (99%, TFA), trichloroacetic acid (99%, TCA), $CaCl_2$, Na_2SO_4, $BaCl_2$, arabinose, rhamnose, D-galactose and L-fucose were from Sigma–Aldrich Co. (Steinheim, Germany). Agarose D-2 was obtained from Hispanagar (Burgos, Spain). Caspase-3 colorimetric assay kit was obtained from Biovision, Inc. (Mountain View, CA. USA). Minimal essential medium eagle (MEM-eagle) cell culture media was purchased from Sigma–Aldrich Co. (Steinheim, Germany); foetal bovine serum (FBS) was from Flow Laboratories (North Ryde, N.S.W., Australia); streptomycin–penicillin and Trypan Blue were from Gibco (Canada). Cell Proliferation Kit 1 was obtained from Roche Applied Science, Germany. The PE Annexin V Apoptosis Detection Kit 1 was obtained from BD Biosciences (Franklin Lakes, NJ, USA). All chemicals used were analytical grade.

4.2. Extraction of FCSPs from S. henslowianum *C. Agardh*

The *Sargassum* FCSP product (FSAR) used was extracted from *S. henslowianum* C. Agardh by use of an optimized single-step extraction procedure described previously [6]. Briefly, the dried *S. henslowianum* seaweed was ground and sieved to pass through a 500 μm sieve and 100 g of dried ground seaweed was extracted in 2 L of 0.03 M HCl with continuous stirring at 200 rpm for 4 h at 90 °C water bath (Julabo, Germany). The suspended seaweed was filtered, and the extract was precipitated using 60% ethanol, the precipitate collected after centrifugation at 10,600 rpm for 10 min (Sigma Laboratory Centrifuge 4K15, VWR, Denmark), and the resulting pellet was freeze dried. This freeze dried pellet constituted the fucose-containing sulfated polysaccharides (FSAR).

4.3. Acid Hydrolysis and FCSPs Composition Analysis

The freeze dried FSAR and FVES samples (20 mg) were hydrolyzed separately in 2 M TFA (final concentration) at 121 °C for 2 h, then the hydrolyzed mixture were freeze dried at −57 °C (Heto Lyolab 3000, England). Each dried powder sample was resolubilized in doubly distilled water and centrifuged at 10,000 rpm for 10 min to collect the supernatant (Sigma Laboratory Centrifuge 4K15, VWR, Denmark). Each supernatant was filtered through a 0.2 µm syringe tip filter (SUN-Sri, Rockwood, TN) prior to injection into the HPAEC-PAD for monosaccharide analysis [41]. Analysis of sulfate content was done according to the method described by Jackson and McCandless [42].

4.4. ^{1}H NMR and FTIR Spectroscopy

The ^{1}H NMR spectra were obtained using an INOVA 600 NMR spectrometer (Agilent Technologies Japan, Ltd., Tokyo Japan) equipped with a ^{1}H[^{15}N-^{31}P] pulse field gradient indirect-detecting probe. Standard pulse sequences were used in all operations. The ^{1}H chemical shift (δH) was referenced to HOD (δH 4.76 ppm, $^{2}H_2O$). The ^{1}H NMR spectrum was assigned through the ^{1}H–^{1}H decoupling technique. An NMR spectrum of L-fucose was utilized as a reference for chemical shift assignment. The lyophilized FCSPs powders were dissolved in deuterium oxide ($^{2}H_2O$) and evaporated to exchange the unstable ^{1}H with ^{2}H. The evaporation and dissolution step was repeated five times, and the samples (10 mg) were finally dissolved in 0.75 mL $^{2}H_2O$ and then subjected to NMR spectroscopy. The IR spectra were obtained using a Spectrum One FT-IR spectrometer (Perkin Elmer, Waltham, MA, USA) equipped with universal attenuated total reflectance (UATR) accessories. Analysis of each of the FSAR and FVES powders, ~1 mg of each, was done using diffuse reflectance infrared transform spectroscopy (DRIFTS) and the spectrum was evaluated by Perkin Elmer Spectrum software version 5 (Perkin Elmer, Waltham, MA, USA).

4.5. Cell Culture and Anti-Proliferative Assay

Melanoma B16 cells (MC) were grown in MEM eagle medium supplemented with 10% (v/v) heat inactivated FBS, 1% (w/v) streptomycin–penicillin and 1% (v/v) of 200 mM L-glutamine at 37 °C under 5% CO_2. Monolayer cultivation was carried out by adding 100 µL of the cell-MEM-FBS mixture into separate wells in 96-flat well plates at a density of 6×10^4 cells per well followed by incubation for 24 h in 5% CO_2 at 37 °C. For the anti-proliferation assay the medium was removed after the 24 h of monolayer cell cultivation and replaced with 100 µL of MEM medium containing 2% FBS and varying concentrations (0.1–1.0 mg/mL) of the crude FCSPs, *i.e.*, FSAR and FVES, respectively, and the mixtures were then incubated for 24 h. Quantification of cell proliferation was carried out using a tetrazolium salt (MTT (3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide)) based colorimetric assay following the protocol supplied with the Cell Proliferation Kit 1 (Roche Applied Science, Germany). Briefly, 20 µL MTT solution (5 mg/mL) was added to the cell cultures after the 24 h of incubation with the FCSPs, and the cell cultures were then re-incubated for 4 h. Finally, 100 µL of stabilization solution was added to each well and the plates were incubated overnight at 37 °C under 5% CO_2. Absorbance was measured using an Elisa reader at 550–690 nm.

4.6. Cell Culture and Caspase-3 Assay

Melanoma B16 cells (MC) were grown in MEM eagle culture medium supplemented with 10% (v/v) heat inactivated FBS, 1% (w/v) streptomycin–penicillin and 1% (v/v) of 200 mM L-glutamine maintained at 37 °C under 5% CO_2. For the caspase-3 assay, monolayer cultivation was carried out in a petri dish (60 × 15 mm) by adding 5 mL culture medium containing melanoma cells at a density of 1×10^5 per mL and varying concentrations (0.2, 0.4 and 0.8 mg/mL) of the FSAR and FVES, respectively. The mixtures were then incubated for 24 and 48 h in 5% CO_2 at 37 °C. The caspase-3 assay was performed according to the protocol supplied with the assay kit (Biovision Inc., Mountain View, CA, USA) used to assay the activity of caspases that recognize the amino acid sequence DEVD. The assay was based on spectrophotometric detection of the chromophore *p*-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. Concisely, the melanoma B16 cells exposed to FSAR and FVES, respectively, were harvested and resuspended in 50 µL of cell lysis buffer and incubated on ice for 10 min. and the mixture centrifuged for 1 min (14,000 × *g*, 4 °C). Each supernatant was then transferred to a fresh tube, and reaction buffer (50 µL) and 4 mM DEVD-pNA substrate (5 µL) were added, and this reaction mixture was then incubated at 37 °C for 1 h. Absorbance of pNA light emission was quantified using a microtiter plate reader at 405 nm.

4.7. Apoptosis Assay by Fluorescence-Activated Cell Sorting (FACS)

After 24 h of monolayer cultivation of melanoma B16 cells with 0.2 mg/mL of FSAR or FVES, and no FCSPs addition as control, the culture medium was removed, and the cells harvested by addition of 1 mL Trypsin-EDTA. The harvested cells were washed twice with 0.1 M PBS and then resuspended in binding buffer according to the protocol for the Annexin V Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA). 100 µL of this solution at 1×10^5 cells was transferred to a culture tube and 5 µL of Annexin V and 5 µL of 7-amino-actinomycin (7-ADD) were added, and the mixture incubated at room temperature for 25 min. Then, 400 µL of binding buffer was added and the extent of apoptosis and staining pattern of the cells were tracked by flow cytometric analysis on a FACScan instrument (Becton Dickinson).

5. Conclusions

The tumor inhibitory bioactivity of fucose-containing sulfated polysaccharides (FCSPs) from *Sargassum henslowianum* C. Agardh (FSAR) and *F. vesiculosus* (FVES) was demonstrated through evaluation of inhibition of melanoma cell proliferation, activation of caspase-3, and apoptosis of melanoma B-16 cells *in vitro*. The structural traits of the FCSPs products were shown to be complex and to differ among the two FCSPs making it delicate to draw definite conclusions about structural effects and mechanisms. However, since the sulfate levels were relatively high as well as relatively similar among the two FCSPs, we propose that the bioactivity effects of the FSAR and FVES might be attributable to the sulfation (charge density), positioning and bonding of the sulfate substitutions in the FCSPs. The work clearly indicates that unfractionated fucose-containing sulfated polysaccharides from both *Sargassum henslowianum* C. Agardh and *Fucus vesiculosus* may have therapeutic potential as skin-cancer preventive agents.

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Samples Availability: Available from the authors.

5 Seaweed nutrient assimilation and growth response

Industrial sewage, fish farm effluents, and agricultural runoff are the major causes of eutrophication of aquatic systems. Eutrophication has adverse environmental effects including hypoxia; on the other hand, some aquatic organisms may experience increased population, promoted growth, and the production of algae, including fast-growing seaweed. The use of large-scale seaweed populations is thought to provide new and cost-effective technologies for sequestering and preventing the distribution of waterborne pollutants. Seaweeds can remove several pollutants through assimilation, bioaccumulation, and detoxification. Integration of seaweed cultivation with fish aquaculture has been proposed to reduce the nutrient burden of fish effluents (Chopin et al., 2001). *Ulva* sp. has been considered the integrated biofilter system and has shown reasonably high efficiency in the removal of waste inorganic nutrients (Chung et al., 2002). Seaweed accumulates a wide range of heavy metals regardless of radioactivity status. In highly polluted seawaters, bioaccumulation can be as high as 1 g per dry gram of seaweed tissue, while approximately 5–100 mg/g in normal seawaters (Burdin and Bird, 1994; Grüven et al., 1993).

Opportunistic green macroalgae such as *U. lactuca* can rapidly consume the available inorganic nutrients at a rate that depend on the availability of ammonium and nitrate (Pedersen and Borum, 1996) and, hence, influence their differential growth responses. *U. lactuca* has a high growth potential doubling time of approximately 2 days with a production potential of 45 T (total solids) ha^{-1} yr^{-1} (Christensen and Sand-Jensen, 1990; Bruhn et al., 2011). Nitrogen enrichment studies have shown uptake rates and growth responses that were more in favor of ammonium than nitrate since they require energy-demanding nitrate reductions (Pedersen and Borum, 1996; Solomonson and Barber, 1990). *U. lactuca* was known to have high affinity for dissolved inorganic carbon (DIC). Rapid photosynthesis in dense floating mats leads to DIC depletion and increases pH and oxygen levels; as a consequence, these changes may all inhibit carbon fixation and, thus, reduce growth. Photosynthetic experiments have shown that the growth response of *Ulva* species was weakly affected by oxygen levels, whereas pH approaching 10 was highly inhibitory (Christensen and Sand-Jensen, 1990).

5.1 Evaluation of growth and assimilation

Quantitative determination and accurate measurement of productivity responses are essential aspects for understanding the primary production and the response to different transient nutrients. The common method and technique to determine seaweed growths in culture systems are gravimetric, and direct contact to the algal tissue when removing of surface moisture (Msuya et al., 2008). Measurement of the surface area of circular disks from algal thalli has been used in some growth monitoring studies (Sand-Jensen, 1988; Christensen and Sand-Jensen, 1990). The traditional method for determining uptake rates has been to measure changes in nitrogen concentration in the incubation medium over time (Harlin and Wheeler, 1985). More recently, incorporation of the nitrogen-stable isotope ^{15}N into algal tissue has been used to calculate uptake rates (Naldi and Wheeler, 2002). Specific growth rates were mostly expressed in percentage day^{-1}, while uptake rates were expressed as µM N g Dw^{-1} day^{-1}. The uptake rate was calculated using the changes in ammonium and nitrate concentration during each sampling interval according to $V= [(S_0 \times vol_0) - (Si - vol_i)]/(t \times B)$, where S_0 is the ammonium concentration, vol_0 is the water volume at the beginning of a sampling interval, S_i is the ammonium concentration, vol_i is the water volume at the end of the sampling interval, t is the time elapsed between 2 successive samplings, and B is the amount of biomass DW (Pedersen, 1994). A specific growth rate (µ) as a percent increase in disc area expansion was calculated according to $\mu = 100 \times Ln (A_i/A_0)/t$, where A_0 is the initial disc area and A_i is the disc area at time t (DeBoer et al., 1978).

5.2 Growth response of *U. lactuca*

This section is an extended elucidation of Paper 1: differential growth response of *U. lactuca* to ammonium and nitrate assimilation.

5.2.1 Relevance

Increased fluctuating levels of nutrients in the estuarine ecosystem stimulate the abundance and production of fast-growing algae like the ephemeral macroalgae *U. lactuca* (Twilley et al., 1985). The abundant growth of ephemeral macroalgae is so invasive that it could lead to oxygen depletion and shading among other marine habitats. Enormous quantities of this seaweed create environmental concerns (e.g., microbial waste and rancid odors) when cast away on the beach. *U. lactuca* a common ephemeral macroalga from the tropical to polar climates that has been harvested from natural populations or cultivated in land-based systems and as part of integrated multi-trophic aquaculture systems. Nevertheless, most of today's *U. lactuca* biomass is unused,

dumped or left stranded to decompose in the shore creating waste problem (Morand et al., 2006). Hence, utilization of *U. lactuca* is of ardent importance.

Production of alternative biofuels from non-starch biomass has directed attention to the utilization of *U. lactuca* as primary substrates for anaerobic digestion to biogas. Moreover, *Ulva* species are rich in rare cell wall polysaccharides and vitamins A, B2, B12, and C, and they exert antioxidant, antimicrobial, and antiviral activities (Ivanova et al., 1993; Abd El-Baky et al., 2008; Ortiz et al., 2006). For this reason, cultivation of *U. lactuca* for either crude biomass production or for the production of biologically active compounds is currently receiving increased attention (Hiraoka and Oka, 2008). Nonetheless, a major prerequisite for the successful exploitation of cultivated *U. lactuca* for commercial applications is that both the growth rate and the yield are optimized. This in turn requires both an understanding of the influence of different nutrients on the growth response and a precise methodology to measure the growth.

5.2.2 Hypotheses and objectives

The utilization of ammonium and nitrate by seaweed varies among species, and the assimilation of these nutrients influences the growth. It has been observed that the presence of ammonium inhibits nitrate uptake (Thomas and Harrison, 1987). These interactions, however, were only discussed in relation to nitrogen uptake rates and the understanding of the influence of these interactions on seaweed growth responses, including those of *U. lactuca*, remains limited. The common method to determine growth rate and biomass yield in seaweed culture systems involves determination of the initial and final weight of seaweed samples. This requires removal of surface moisture either by wiping with a filter cloth or by centrifugation. The extent of remaining surface moisture induces varying degrees of measurement inaccuracy (Msuya and Neori, 2008; Vandermeulen and Gordin, 1990). In continuous culture monitoring, the harvested seaweeds are dehydrated prior to weighing, and then a stock strain is taken and used as the initial inoculum material for further culture. However, seaweed needs to recuperate from the harsh dehydration-hydration treatment before it can resume growth. Therefore, the interruption of cellular growth is almost unavoidable (Phillips et al., 2002).

Therefore, the objective of this work was to evaluate the growth response of *U. lactuca* cultured in artificial seawater exposed to different sources and levels of nitrogen (NH_4^+ and NO_3^-) using a more accurate growth monitoring technique. To achieve the precise evaluation, a photo-scanning technology was used to obtain digital images of the sizes of the frond discs, and in this way

examined the growth kinetics by measuring the surface area expansion of the seaweed discs using digital image processing software.

5.2.3 Result highlights

The growth response of *U. lactuca* exposed to different sources of nitrogen (NH_4^+, NO_3^-, and the combination NH_4NO_3) was examined using photo-scanning technology. Differential increases of the surface area of *U. lactuca* discs with time in response to different N-nutrient enrichments were expected. The NH_4Cl- and $NaNO_3$-rich media (50 µM of N) accelerated *U. lactuca* growth to a maximum specific growth rate of 16.4 ± 0.18% d^{-1} and 9.4 ± 0.72% d^{-1}, respectively. The highest obtained biomass production was 22.6 ± 0.24 mg-DW m^{-2} d^{-1}. The *U. lactuca* growth response favored ammonium as the nitrogen source, and its presence apparently discriminated nitrate uptake by *U. lactuca* when exposed to NH_4NO_3.

Apart from showing a significant differential growth response of *U. lactuca* to different nitrogen sources, the work exhibits the applicability of a photo-scanning approach for acquiring precise quantitative growth data for *U. lactuca* as exemplified by assessment of the growth response to two different N-sources.

5.2.4 Consideration and justification

The exploitation of land-based resources for bioenergy production created global concern for its impact on the food production, availability, and price of commodities. Meanwhile, the utilization of marine-based resources, notably non-commercially important seaweed, is gaining attention. This leads macroalgae like *U. lactuca* to be considered for the production of bioenergy because it exhibits production potential for either land-based culture or off-shore cultivation (Bruhn et al., 2011). Furthermore, other potential applications of *U. lactuca* biomass (e.g., source of functional and bioactive compounds) must be established to add more value to the seaweed biomass.

Nevertheless, the growth rate, yields, and culture condition must primarily be optimized to successfully exploit *U. lactuca* for commercial applications. Therefore, understanding the effect of different nutrients on the growth response is critical, and it requires precise methodology to measure the growth with the purpose of effectively evaluating nutrient assimilation.

Over the growth experiment duration, an artificial seawater medium was used without renewal, which may have influenced the growth kinetics of the algal frond. However, the results indicate that *U. lactuca* can grow even without renewing the artificial seawater medium for a certain period. The maximum growth rate was close to that of a published study (Neori et al., 1991),

where they used fresh seawater medium and extensive medium renewal. However, the amount of biomass accumulated during this experiment was relatively low compared to those of other earlier works (Neori et al., 1991). This variation was probably attributed to different monitoring techniques and culture conditions, such as water flow velocity, temperature, density, and illumination intensity.

5.3 Paper 5: Differential growth response of *Ulva lactuca* to ammonium and nitrate assimilation

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Differential growth response of *Ulva lactuca* to ammonium and nitrate assimilation

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Abstract Controlled cultivation of marine macroalgal biomass such as *Ulva* species, notably *Ulva lactuca*, is currently studied for production of biofuels or functional food ingredients. In a eutrophic environment, this macrophyte is exposed to varying types of nutrient supply, including different and fluctuating levels of nitrogen sources. Our understanding of the influences of this varying condition on the uptake and growth responses of *U. lactuca* is limited. In this present work, we examined the growth response of *U. lactuca* exposed to different sources of nitrogen (NH_4^+; NO_3^-; and the combination NH_4NO_3) by using photo-scanning technology for monitoring the growth kinetics of *U. lactuca*. The images revealed differential increases of the surface area of *U. lactuca* disks with time in response to different N-nutrient enrichments. The results showed a favorable growth response to ammonium as the nitrogen source. The NH_4Cl and $NaNO_3$ rich media (50 μM of N) accelerated *U. lactuca* growth to a maximum specific growth rate of 16.4±0.18% day^{-1} and 9.4±0.72% day^{-1}, respectively. The highest biomass production rate obtained was 22.5±0.24 mg DW $m^{-2}{\cdot}day^{-1}$. The presence of ammonium apparently discriminated the nitrate uptake by *U. lactuca* when exposed to NH_4NO_3. Apart from showing the significant differential growth response of *U. lactuca* to different nitrogen sources, the work exhibits the applicability of a photo-scanning approach for acquiring precise quantitative growth data for *U. lactuca* as exemplified by assessment of the growth response to two different N-sources.

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Keywords Ammonium · Nitrate · Growth monitoring · Seaweed cultivation · *Ulva* · Nutrient uptake

Introduction

Ulva lactuca is an important macroalga in marine ecology. Its fronds are soft, sheet-like structures that are two cells thick, and this morphology is the reason for its common name "sea lettuce". Recently, production of alternative fuels from non-starch biomass has also directed the attention to utilization of marine algae, including seaweed or macroalgae, as sources of biomass for biofuels production (Knauf and Moniruzzaman 2004). The rapid growth of *U. lactuca* is attributed to its high photosynthetic rates and high C and N-nutrient uptake capacity (Magnusson et al. 1996; Naldi and Wheeler 2002; Sand-Jensen 1988). Seaweed has also been proposed as a biomass source for production of functional food ingredients, pharmaceuticals, and cosmetics (Bodin-Dubigeon et al. 1997; Cumashi et al. 2007; Bixler 1996; De Roeck-Holtzhauer 1991). *Ulva* species are particularly rich in rare cell-wall polysaccharides and have been proposed as being an important source of dietary fiber, mainly soluble fiber (Lahaye 1991; Lahaye and Axelos 1993). *Ulva lactuca* is also a good source of Vitamin A, B2, B12, and C and is rich in γ-tocopherol and *U. lactuca* extracts have been shown to exert antioxidant, anti-microbial, and anti-viral activities in various in vitro assays (Ivanova et al. 1994; Abd El-Baky et al. 2008; Ortiz et al. 2006).

It has been shown previously, that *U. lactuca* is suitable for propagation under controlled conditions (Vermaat and Sand-Jensen 1987; Lee 2000; Sato et al. 2006a, b). For this reason, cultivation of *U. lactuca* in tanks for either crude biomass production or for production of biologically active compounds is currently receiving increased attention

(Hiraoka and Oka 2008). However, a major prerequisite for successful exploitation of cultivated *U. lactuca* for commercial applications is that the growth rate and yields are optimized. This in turn requires both an understanding of the influence of different nutrients on the growth response and a precise methodology to measure the growth.

Ammonium and nitrate are the major nitrogen sources of seaweeds in the natural habitat and are used as nourishment for seaweed in integrated multi-trophic aquaculture systems (Copertino et al. 2008). Several experiments have been conducted for several seaweed species on the interaction of ammonium and nitrate uptake. The utilization of ammonium and nitrate by seaweed varies among species and assimilation of these nutrients influences the growth. It has been observed that the presence of ammonium inhibits the nitrate uptake; thus ammonium uptake dominated nitrate uptake when exposed to a combination of NH_4^+ and NO_3^- (Thomas and Harrison 1987). However, these interactions were discussed only in relation to nitrogen uptake rates and the understanding of the influence of these interactions on seaweed growth responses, including growth responses of *U. lactuca*, is still limited. The common method to determine growth rate and biomass yield in seaweed culture systems involves determination of the initial and final weight of seaweed samples. This requires removal of surface moisture either by wiping with filter cloth or by centrifugation. The extent of remaining surface moisture induces varying degrees of inaccuracy to the measurement (Msuya and Neori 2008; Vandermeulen and Gordin 1990).

In this present work, the objective is to evaluate the growth response of *U. lactuca* exposed to different sources of nitrogen (NH_4^+ and NO_3^-). We cultured *U. lactuca* frond disks for 10 days in small containers with artificial seawater (ASW) enriched with equimolar levels of nitrogen (50 μM) of two different N-nutrients, NH_4Cl and $NaNO_3$, to assess the growth responses. Another experiment was conducted in the same set-up for 4 days to evaluate the ammonium and nitrate uptake rates when *U. lactuca* was exposed to 50 μM NH_4NO_3. We used photo-scanning to obtain digital images of the sizes of the frond disks, and in this way examined the growth kinetics by measuring the surface area expansion of the seaweed disks daily using commercially available digital image processing software.

Materials and methods

The chemicals used for nitrogen source were NH_4Cl (99.5%), NH_4NO_3 (99.5%), and $NaNO_3$ (99.0%), purchased from Sigma-Aldrich, (Bornem, Belgium). Commercial marine sea salt (Sera Marine Basic Salt—Heinsberg, Germany) was used to prepare artificial seawater (ASW) principally as described by Sato et al. (2006a, b). For nitrate determination, a low-range lab nitrate test kit supplied with nitrate reductase was used (The Nitrate Elimination Co., Inc., Michigan).

Aquarium culture: condition and set-up

Fresh *Ulva lactuca* fronds were obtained from the National Environmental Research Institute of Denmark (DMI), University of Aarhus (Silkeborg, Denmark). The original seaweed material was cultured in ponds with natural seawater. Upon arrival to our laboratory, the seaweed fronds were transferred into a 2 L beaker containing 1.5 L ASW with a 33 g L^{-1} concentration of marine sea salt to habituate. The ASW medium had a pH of 8.35 and 21.5 ppt salinity measured using a handheld conductivity meter (Con 11, Eutech Instruments—Singapore). The fronds were acclimatized in the beaker with aeration for 5 days prior to transfer into a 112-L aquarium tank for long term cultivation. The water at ambient temperature in the 112-L aquarium was led directly to a 45-L reservoir tank and the water was then pumped back to the aquarium at a rate of approximately 1,700 L h^{-1}. The reservoir was aerated and the water (ASW) was renewed weekly.

Flask culture set-up

The growth monitoring of the *U. lactuca* disks during differential cultivation was carried out using a perforated flask (500 mL) positioned inside a rectangular canister that served as a catchment basin. The culture flask was perforated at a certain height to allow the medium to overflow after reaching the 300 mL level into the catchment basin. The medium was then re-circulated at a speed of 1.5 L h^{-1} using a peristaltic pump. A small magnetic bar was placed at the bottom of the flask to mix the water twice daily and to ensure that the nutrients were properly distributed. To prevent the contact of the magnetic bar and the *U. lactuca* disks, a 5-mm mesh was fitted inside in the middle of the flask. A schematic diagram is shown in Fig. 1. The media in the individual flasks were enriched with 50 μM NH_4Cl, 50 μM $NaNO_3$ and ASW as control, respectively. A 5-mL water sample was collected daily to assess the ammonium and nitrate uptake of *U. lactuca*. From this set-up, two separate cultivation experiments were conducted, a 10-day cultivation period was used for growth monitoring while a 4-day cultivation was used to evaluate the nitrogen uptake response of *U. lactuca* to the interaction of equimolar ammonium and nitrate supply (50 μM NH_4NO_3).

Nitrogen uptake measurement

Ammonium concentration was determined with the endophenol-blue method (Koroleff 1970). Nitrate measurement was conducted using low-range lab nitrate test kit

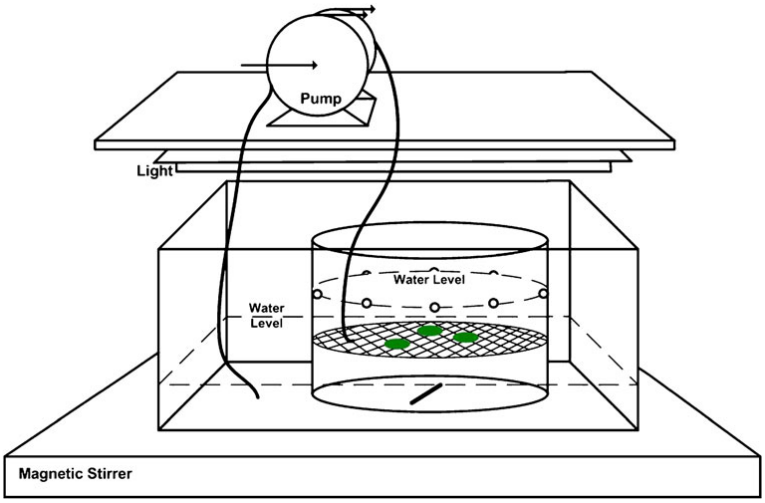

Fig. 1 Schematic set-up of a laboratory seaweed cultivation system utilized for the growth monitoring of *U. lactuca*

supplied with nitrate reductase as described by Campbell et al. (2004). The uptake rate was calculated from changes in ammonium and nitrate concentration during each sampling interval according to $V = [(S_0 \times \mathrm{vol}_0) - (S_i \times \mathrm{vol}_i)]/(t \times B)$ where S_0 is the ammonium concentration, vol_0 is the water volume at the beginning of a sampling interval; S_i is the ammonium concentration, vol_i is the water volume at the end of the sampling interval, t is the time elapsed between two successive samplings and B is the amount of biomass dry weight (DW) (Pedersen 1994). Throughout the experiment the *U. lactuca* disks were illuminated with a parallel 2X18W Lumilux Cool White light (Osram—Germany) from a fluorescent lamp which was placed 12 cm above the top of the culture flask, light and dark ratio was 14:10 hours. Illumination at a continuous irradiance of 56 μmol photons m^{-2} s^{-1} was measured using a handheld Field Scout quantum light meter (Spectrum Technologies, Inc., USA). The size expansion of each seaweed disk was monitored daily using scanning and image processing software as described below.

Scanning, imaging, and growth measurements

Fresh and healthy fronds were collected from the aquarium, and then an approximately 9 mm diameter sharpened pipette tube was punched randomly into different *Ulva* fronds to form a disk. All disks were scanned and the area was measured prior to inoculation. Three *U. lactuca* disks were inoculated in the flask with ASW medium. Before scanning, all disks were carefully transferred into a petri dish containing a small volume of the medium to prevent the seaweed disk from drying. Microscope glass slides were used to cover the seaweed disk to ensure that the entire surface was plane, and then the disks were scanned using a CanoScan 5600F (Canon) at 300 dpi resolution equipped with MP Navigator Ex software (Canon) for digital imaging. The images were calibrated by cropping them at 200×200 pixels using Photoscape v3.3 image editor (Mooii—Seoul, Korea) prior to disk area measurement. Image-Pro Plus software (Media Cybernetics, Inc., USA) was calibrated appropriately for image analysis and measurement of the size/area of the image in mm^2. This was done by scanning a ruler at 300 dpi, and then the image was cropped at 200×200 pixels. A length of 1 mm was measured via a ruler image, saved, and then used as the standard for disk image calibration.

Disk area growth, specific growth rates, and biomass yield

After measuring the initial area of all the disks, three disks with known area were randomly collected, weighed and subjected to drying in a 105°C oven for 4 h and then the disk dry weight was obtained. The measured dry weight (DW) was used to convert the disk area into dry weight, resulting in a conversion factor of 0.027 mg DW mm^{-2}. The equivalent seaweed DW of the disk was used to calculate the biomass produced as the sum of the DW of all the disks (mg) on the culture flask per culture area of the flask (0.065 m^2) per unit time (day) expressed in units of mg m^{-2} day^{-1}. The disk area growth was calculated according to this equation $A_G=[(A_i-A_0)/(A_c\times t)]$ where A_G is the disk area growth (mm^2 m^{-2} day^{-1}), A_i is the disk area (mm^2) at the end of sampling interval, A_0 is the disk area (mm^2) at the beginning of the sampling interval, A_c is the area of the culture flask (0.065 m^2) and t is the time (day) elapsed between two successive samplings. Specific growth rate (μ) as percent increase in disk areal expansion was

calculated according to $\mu=100\cdot\mathrm{Ln}\,(A_i/A_0)/t$ where A_0 is the initial disk area and A_i is the disk area at time t (DeBoer et al. 1978). Statistical analysis was performed using Proc GLM and Proc Mixed of SAS Statistical Software version 9.1 (SAS Institute, Inc., USA) and graphs were made using Sigma Plot 10.0 (Systat Software Inc., USA).

Results

Cultivation, imaging, and growth measurement

Disks prepared from *U. lactuca* fronds grew steadily and reproducibly. The differences in *U. lactuca* disk area growth induced by the different N-enrichment (no N-addition, 50 μM NH_4Cl or 50 μM $NaNO_3$) was monitored daily throughout the cultivation period. Images of the disk fronds presented in Fig. 2 exhibit the increase in the surface area of *U. lactuca* disks over time with the different nitrogen sources. The fronds that were enriched with NH_4Cl and $NaNO_3$ produced an olive-green color, which may be a result of the accumulation of chlorophyll in the fronds. The color was slightly more yellow-green but also denser in the case of ASW-grown fronds (Fig. 2). The color differences may result from difference of nutrients. The ammonium-enriched medium induced a significantly higher increase ($P< 0.05$) in surface area expansion (148.8 mm m^{-2} day^{-1}) than the nitrate-enriched medium (98.6 mm m^{-2} day^{-1}) , while the disks incubated in the ASW without N-enrichment grew only slowly with no significant area expansion during the 10 days of cultivation (Fig. 2). Nitrate availability in the medium contributed little to the growth but nevertheless the disk area expansion after 10 days was significantly higher ($P<0.05$) with the nitrate-enriched medium than that obtained with ASW only.

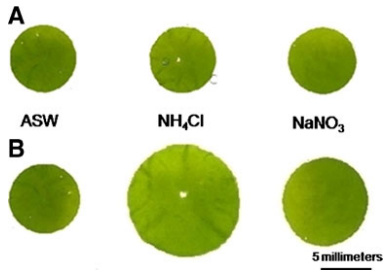

Fig. 2 The growth difference of the surface area in the images **a** Day-1 and, **b** Day-10 culture in artificial sea water (*ASW*) enriched with different ammonium and nitrate nutrients. Images were generated after photo-scanning using CanoScan 5600F scanner at 300 dpi resolution

Biomass yield and specific growth rate

Both ammonium- and nitrate enrichment elicited a normal, hyperbolic growth response as depicted in the accumulation of biomass (Fig. 3). On the NH_4^+-enriched medium the biomass yield showed a steep increase during days 2–5 whereas the yield varied much less during the first days for the NO_3^- growth. The difference in the disk growth in response to the type of N-source was noticed from the fifth day of cultivation onward (Fig. 3). The growth increment (specific growth rate) stalled after about 8 days of cultivation, presumably as a result of nitrogen limitation in the media. The specific growth rate increased with substantial amount of NH_4 and stabilized after the nutrient was depleted. The maximum specific growth rate of *U. lactuca* cultured on NH_4^+ was 16.4± 0.18% day^{-1} and 9.4±0.72% day^{-1} with NO_3^- during 10 days of cultivation. The growth kinetics on nitrate illustrated a modest growth response as exemplified by the biomass yield of *U. lactuca* on the nitrate nutrient source relative to that on the NH_4^+ (Fig. 3). Ammonium increased the biomass yield significantly ($P<0.05$) relative to the NO_3^- nutrient. The maximum biomass yield was thus 22.5±0.24 mg m^{-2} day^{-1} by ammonium and only 13.0±0.40 mg m^{-2} day^{-1} by nitrate enrichment.

Nitrogen uptake and interaction

Further analysis of the nutrient uptake response of *U. lactuca* in relation to different nitrogen source showed that ammonium was favorably assimilated corresponding to a high uptake rate of ammonium (Fig. 4a, b). Both ammonium and nitrate were assimilated as indicated by the decrease of concentration in the medium (Fig. 4a). Significant differences ($P<0.05$) were observed for both the concentration and uptake rate of ammonium and nitrate over time (Fig. 4a, b). The abrupt assimilation of ammonium with high uptake rate from 0 to 1 day of cultivation was probably due to starvation of the seaweed frond. Nitrate in the medium was slowly assimilated by *U. lactuca* until an increased uptake rate was observed between 3 and 4 days of cultivation (Fig. 4b). The *U. lactuca* disks were also exposed to 50 μM NH_4NO_3 in order to evaluate the uptake response of *Ulva* to the combination of the two nitrogen sources. This combination of ammonium and nitrate in the medium demonstrated the interactive response of *U. lactuca* to these nutrients (Fig. 4c). This interaction showed that the simultaneous presence of ammonium and nitrate resulted in an NH_4^+ uptake rate which was similar to that of the NH_4^+ uptake rate, when exposed to ammonium alone, but a relatively *higher* uptake rate of NO_3^- as compared to when exposed to $NaNO_3$ alone (compare Fig. 4 b and c). However, the ammonium uptake rate was still significantly higher than

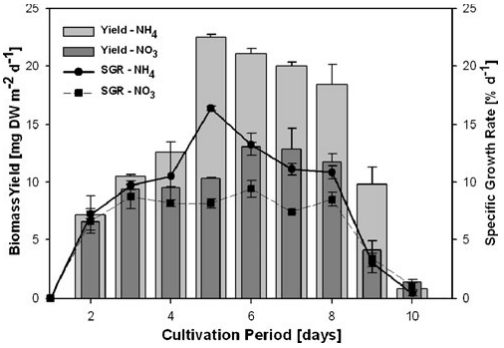

Fig. 3 Biomass yield (*Y* axis *scale to the left*) and specific growth rate (SGR, *Y* axis *scale to the righ*) of *U. lactuca* during 10-days of cultivation. The maximum specific growth rate was 16.4± 0.18%·day^{-1} assimilated from NH_4 and 9.4±0.72%·day^{-1} from NO_3. Initial ammonium and nitrate concentrations were 50 μM NH_4Cl and 50 μM $NaNO_3$. Maximum biomass yield were 22.5±0.24 mg DW m^{-2} day^{-1} (day 5) by ammonium and 13.0±0.40 mg DW m^{-2} day^{-1} (day 6) by nitrate enrichment

the nitrate uptake rate (Fig. 4c). The data indicate that the nature of assimilation of nitrogen by *U. lactuca* is selective.

Discussion

Our understanding of the growth kinetics of *U. lactuca* is highly dependent on the precise monitoring of its growth and measurement of the uptake response to different nutrient sources. This includes minimizing the inaccuracy during growth measurement of seaweed fronds. The *U. lactuca* fronds are only two cell layers thick making them vulnerable to damage. By cautiously handling the disks during measurement obtainment of information about growth kinetics could be achieved. In this present work, each individual disk sample was monitored and analyzed consistently. The images of the *U. lactuca* disks shown in Fig. 2 are the actual pictures obtained by the digital scanning. We used commercially readily available image processing software to determine the exact disk expansion. The software used was programmed to automatically count and measure the size of an image provided that the image had been calibrated. Once the parameters, e.g., pixel size, resolution, units, etc. were calibrated, the images were measured uniformly using the same calibration. The experimental set-up was not designed to assess the exact cell growth physiology, which is why no firm conclusion can be drawn as to whether the disk growth is caused by cell division or cell extension. Nevertheless, because of the accuracy of the photo-scanning measurements, the increase in biomass yield could easily be calculated from the increase of seaweed disk area.

Ulva lactuca grew faster with ammonium than with nitrate as the nitrogen source (Fig. 3). This corresponded well with the finding that the uptake of ammonium was faster than the nitrate uptake (Fig. 4b). This difference was probably due to the ammonium being presented in a reduced state which can easily be assimilated and directly utilized by algae for the synthesis of amino acid and protein resulting in cell and tissue growth (Doran 1995). Nitrate, however, must first be reduced to nitrite and then to ammonium in order for the algae to utilize this nitrogen source. The interaction study of the combination of ammonium and nitrate (Fig. 4c) demonstrated the discrimination of ammonium uptake and the uptake of nitrate. According to Lara et al. (1987) the reduction of nitrate to nitrite is catalyzed by the nitrate reductase enzyme that usually uses two ferrodoxins as electrons donors while reduction from nitrite to ammonium is catalyzed by the nitrite

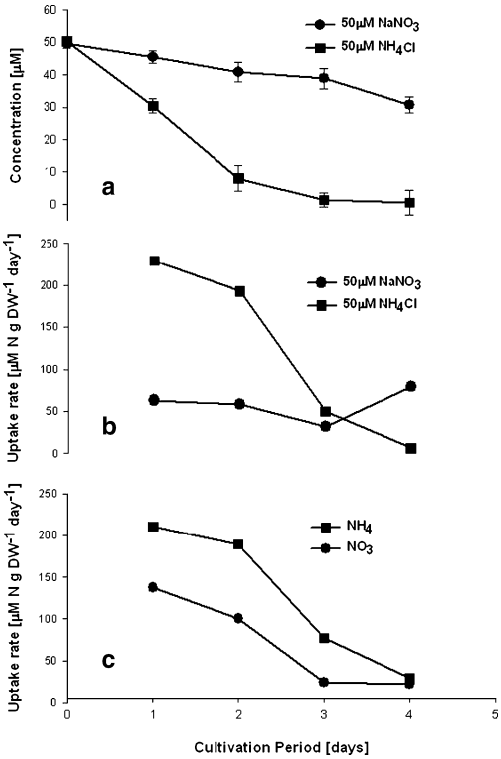

Fig. 4 Four days cultivation of *U. lactuca* in ASW medium enriched with two nitrogen source. **a** Ammonium and nitrate concentration in μM, **b** uptake rate in μM N g Dw^{-1} day^{-1} of ammonium and nitrate when exposed to 50μM NH_4Cl or 50μM $NaNO_3$, **c** uptake rate in μM N g Dw^{-1} day^{-1} of ammonium and nitrate when exposed to 50μM NH_4NO_3; *square* is ammonium uptake rate of and *circle* is nitrate uptake rate

reductase, a process that needs six ferrodoxins. This reduction mechanism as a consequence requires energy for the bioconversion process. Thus, *U. lactuca* growth responses are different given the same concentration of nitrate nitrogen as they would on ammonium nitrogen.

The use of ASW medium over the duration of this experiment without renewal may have influenced the growth kinetics. The common experimental practice in indoor seaweed culture is water renewal (Naldi and Wheeler 2002; Vermaat and Sand-Jensen 1987; Neori et al. 1991; Christensen and Sand-Jensen 1990), however, the effect of the water renewal is unclear. Nevertheless, our results indicate that *U. lactuca* can grow even without renewing the medium for a certain period. The maximum growth rate (μ_{max}) in this condition was 16.39±0.18% day^{-1} that is close (μ_{max}) reported in a previously published study (Neori et al. 1991), where they used fresh seawater medium and extensive renewal of medium.

The amount of biomass accumulated during this experiment was relatively low as compared to other earlier works where they harvested considerable amounts of biomass (Neori et al. 1991; Msuya and Neori 2008). The water flow velocity may be the cause of the relatively lower biomass production. In this work, the water circulation was controlled to be 1.5 L h^{-1}, this circulation was almost equivalent to the water flow in the shallow benthic zone, which should be beneficial for *U. lactuca* growth. This assumption is supported by the previous works of Doty (1971) and Parker (1981) that showed the application of simulated current consistently enhanced growth rates of *U. lactuca* under laboratory conditions. This was also the case in a study conducted by Msuya and Neori (2008) in which they concluded that water velocity affected biomass yields and biofiltration performance of *U. lactuca* under low nutrient concentration in laboratory experiments.

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6 Conclusions and perspectives

As already stated, seaweeds are the subject of a wide range of interesting research topics; therefore, it entails crossing beyond another border of academic discipline to elucidate the problems and accomplish specific objectives. The present study provided information about the most recent developments in the chemistry of fucoidan/FCSPs, emphasizing the significance of different extraction techniques for the structural composition and biological activity using an interdisciplinary approach. The utilization of marine seaweeds that have washed up on the coastline as a source of bioactive compounds like FCSPs and the growth response of *U. lactuca* to nutrient assimilation were thoroughly investigated.

The use of different extraction and purification techniques appears to have contributed to the confusion about the nature and composition of FCSPs ever since it was first described by Kylin early in the 20th century. As detailed in the following, we now know that fucoidan is built of 1→3-linked α-L-fucopyranosyl or of alternating 1→3- and 1→4-linked α-L-fucopyranosyl residues that may be sulfate-substituted, and that some fucoidans isolated from certain brown algae have completely different structures composed of sulfated galactofucans with backbones of (1→6)-linked β-D-galacto- and/or (1→2)-β-D-mannopyranosyl units with (1→3) and/or (1→4)-α-L-fuco-oligosaccharide branching. The available data thus show that the term fucoidan has been used for several different chemical structures and vice versa, that fucoidan is a term that covers a diverse family of FCSPs (Paper 1). It is, therefore, more correct to use the term FCSPs, rather than fucoidan, as a collective term for these polysaccharides.

The varied chemical composition and diverse structures of FCSPs from brown seaweed may have hindered the development of an in-depth understanding of the precise properties of significance for structure-function correlations. Nevertheless, important structural bioactivity issues appear to include the degree of sulfation of the FCSP molecules. Oversulfated FCSPs have thus been found to be excellent potent inhibitors of tumor cell invasion compared to desulfated native FCSPs. Loss of anticoagulant activity has been observed with decreasing degrees of sulfation, although anti-proliferative effects on fibroblast cell lines were retained (Paper 1).

A simple and practical method for recovering a suite of complex FCSPs from *Sargassum* sp. and the effect of different treatment parameters on the integrity of the polysaccharide have been

established (Paper 2). The preservation of the structural integrity of the FCSP molecules appears to be crucial for maintaining its biological properties, and it has been clearly shown that the extraction treatment employed affects the composition and thus the structural features of the FCSP substances (Paper 2). Evidently, the chemical composition and yield of the isolated products are strongly influenced by the method of extraction, as was expected. The data presented (Paper 2) showed that the polysaccharide obtained from a single-step extraction method may be heterogeneous, and that the composition varies with the length of the extraction process. Fucose, sulfate, and glucuronic acid were the important components of the polysaccharide mixture and this is typical for a fucoidan compound (Paper 2). The results (Paper 2) also demonstrated that FCSPs were vulnerable to harsh extraction conditions. Hence, we confirmed that extraction condition significantly influenced FCSP composition, and structural alteration may have occurred. Undoubtedly, the presence of impurities could influence the biological properties of FCSPs and, therefore, may currently hinder our complete understanding of the biological activity of fucoidan or FCSPs. Hence, the development of standard extraction procedures for FCSPs including hydrolysis treatment, purification, and fractionation methodology, preferably with specific steps adapted to the particular botanical order of the seaweed, will generate a better common basis for the analysis and understanding of bioactivities and the mechanisms determining FCSP bioactivities. On this basis, it may even be possible to target specific structural features and, in turn, tune the extraction procedure to obtain specific bioactivities via the use of targeted extraction methodologies.

The bioactivity of the isolated FCSP products from *Sargassum* sp. against LLC and MC was investigated (Paper 3). The study showed that FCSPs induce apoptosis of MC cells (Papers 3 and 4) and exert anti-tumor activity through the inhibition of the growth of LLC and MC, which was probably due to the enhancement of NK cell activity as the principal effector mediating tumor cell death. We showed that both FCSPs samples from *Sargassum* sp. and *F. vesiculosus* induces apoptosis by activating caspase-3 and exerts anti-tumor activity by inhibiting the growth of cells (Paper 4). FCSPs from *Sargassum* sp. and *Fucus vesiculosus* thus appear to be potent against lung and skin cancer cell lines, and its mode of action is associated with the immune response (Paper 3). Furthermore, the bioactivity of crude fucoidan toward these 2 types of cancer cell lines was possibly augmented by the sulfate groups in the fucoidan structure. Nevertheless, further examination about these findings is needed to elucidate the underlying factors of FCSP bioactivity.

The unfractionated FCSP structures from *Sargassum* sp. could probably be heterogeneous and branched as expected; however, another possibility could be that these FCSPs were not mixtures

of different types of polysaccharides but members of the same polysaccharide family. Our structural investigation presented by ^{1}H NMR spectra indicate that in both *Sargassum* sp. and *F. vesiculosus,* FSCPs samples contained fucoidan-like structures, but definite structural information about whether the structure is heterogeneous or members of the same polysaccharide family should further be investigated (Paper 4). The quantitative variation of its components and distribution patterns as well as the differences in its structural details was probably not due to sample heterogeneity but rather to extreme compositional and structural dispersion. At any rate, the crucial bioactive effectiveness of these unfractionated FCSPs may be attributed to their distinct structural features, such as level of sulfation (charge density) and the position and bonding of the sulfate substitutions or sulfated fucans (i.e., C-2 and/or C-4 of α3-linked L-fucopyranose residues) and sulfated galactans (i.e., C-2 of 3-linked galactopyranose residues) complexes (Paper 4). Nonetheless, we now understand that the type and variety of algal FCSPs is much wider than originally believed.

In addition to the investigation of the potential of the brown seaweeds *Sargassum* sp. and *F. vesiculosus* as natural sources of bioactive compounds, the washed up nuisance green seaweed *U. lactuca* was also examined for its growth and nutrient assimilation potential. This study was performed to illustrate the need for a precise monitoring method of the growth of *U. lactuca* in order to successfully exploit it for commercial application. Our understanding of the growth kinetics of *U. lactuca* is highly dependent on the precise monitoring of its growth and measurement of the uptake response to different nutrient sources. This includes minimizing inaccuracies during the growth measurement of seaweed fronds. This work exhibited the applicability of the photo-scanning approach for attaining accurate quantitative growth data of *U. lactuca* as demonstrated by evaluation of the growth response to ammonium and nitrate (Paper 5). The experimental set-up was not designed to assess the exact cell growth physiology, which is why no firm conclusion can be drawn as to whether the *U. lactuca* disc growth was caused by cell division or cell extension. Nevertheless, because of the accuracy of the photo-scanning measurements, the increase in biomass yield could easily be calculated from the increase of seaweed disc area (Paper 5). This result showed that *U. lactuca* grew faster with ammonium than with nitrate as the nitrogen source (Paper 5). This corresponded well with the finding that the ammonium uptake was faster than the nitrate uptake. This difference was probably due to the ammonium being presented in a reduced state that can be easily assimilated and directly utilized by algae for the synthesis of amino acids and proteins resulting in cell and tissue growth. Nitrate, however, must first be reduced to nitrite and then to ammonium for the algae to utilize this

nitrogen source (Paper 5). The interaction study of the combination of ammonium and nitrate demonstrated that the presence of ammonium discriminate the nitrate uptake when *U. lactuca* was exposed to ammonium nitrate. This study provides new technique for evaluating the growth of *U. lactuca* and outlines the possibility of *U. lactuca* as a good agent for bioremediation.

6.1 Future perspectives

Marine seaweed has gained a lot of attention in the scientific community and in industries; thus, the potential biological effects of seaweeds have been exploited has been examined extensively in recent years. Still, there are many avenues in seaweed research, that need to be considered, studied, and understood to successfully exploit this abundant resource. This PhD study highlighted a few of the crucially important factors in the utilization of seaweed products, notably the extraction procedure and bioactivity analysis of FCSPs. Nevertheless, there are other research areas concerning seaweed resources that need further investigation; they may include bioprocesses and purification involving enzyme technology coupled with membrane technology coupled with the concept of seaweed biorefinery.

A typical technology for isolating valuable products from seaweeds, notably bioactive compounds like FCSPs, involves the use of chemicals. This technique has been employed since the first extraction of fucoidan by Kylin in 1913. This present study demonstrated that chemical extraction of fucans from brown seaweeds contained impurities. The apparent impurities (i.e., saccharides other than sulfated fucose) may or may not contribute to the bioactivity efficacy of fucoidan. To reach a definitive conclusion, this hypothesis must be examined strategically using enzymes that cleave specific sites. Extraction using enzymes coupled with membrane technology could be a future strategy for recovering bioactive seaweed compounds. Mono-component activity enzymes may be employed to eliminate and/or hydrolyze specific impurities, including certain monosaccharides and structural polysaccharides. Chemically and/or enzymatically isolated FCSPs compounds can be further purified by filtration using membranes to separate the molecular size of interest. Purified components will be investigated to determine whether they possess any biological active properties against certain diseases using *in vitro, in vivo*, animal models, and/or possibly clinical testing.

Seaweed cultivation and processing is an important industry in Southeast Asia, but it is still new to Scandinavia, especially Denmark. As described in this study, only selected components are obtained from seaweeds (i.e., FCSPs, fucoidan, alginate, carrageenan, and agar), and the remaining are considered waste. This present study proposed a new type of strategy to improve the

utilization of seaweed by using the entire seaweed frond for the production of chemicals and/or biochemicals and energy. Thus, a concept of seaweed biorefinery should be developed.

The first phase of this concept includes isolation of commercially important chemicals/biochemicals such as hydrocolloids and the bioactive sulfated polysaccharide compounds. In the second phase, the remaining residues are collected as bulk biomass and then converted into chemicals and energy carriers (heat, liquid, gas, and electricity) by catalytic and enzymatic conversions and/or by digestion and pyrolysis. The residue-containing minerals are targeted for use in fertilizer applications in the final phase of the biorefinery cycle.

Many potential applications exist for the products acquired from biorefineries. Seaweed components derived thereof can be used as hydrocolloids for food and feed applications or can be formulated into natural health supplements; in contrast, mineral-containing seaweed residues are incorporated into fertilizer products.

The detailed processes, procedures, and specific working conditions of this seaweed biorefinery concept are yet to be determined.

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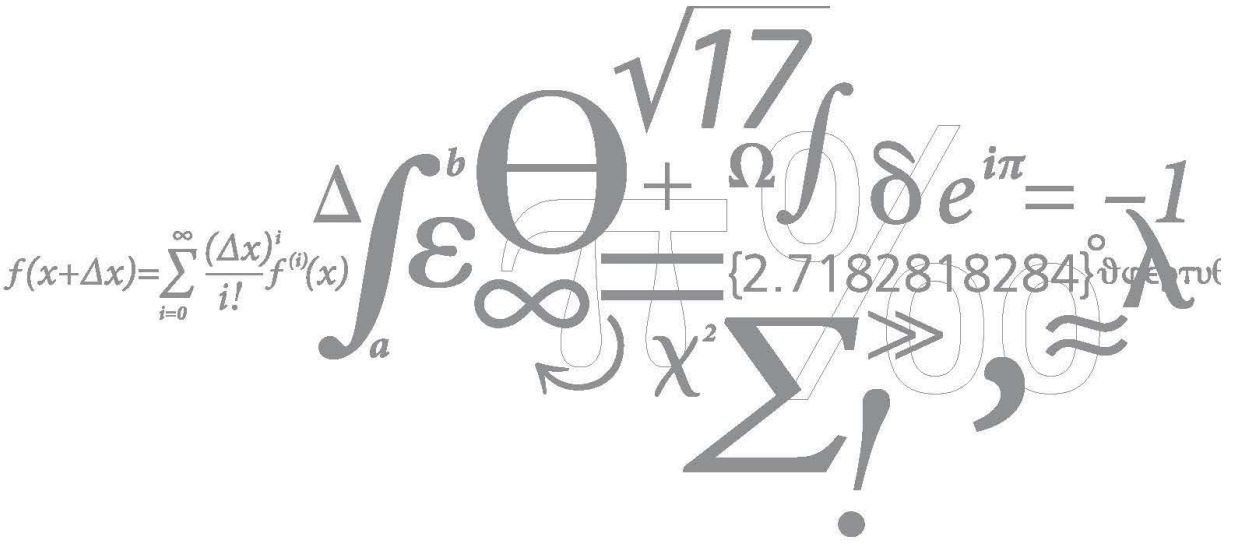
$f(x+\Delta x)=\sum_{i=0}^{\infty}\frac{(\Delta x)^i}{i!}f^{(i)}(x)$
$\int_a^b \varepsilon \Theta + \Omega \int \delta e^{i\pi} = -1$
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$\chi^2 \sum ! \gg \approx \lambda$

In this Doctoral thesis

Fucose containing sulfated polysaccharides (FCSPs) derived from brown seaweed exhibited crucial biological activities including anti-proliferative effects on lung and skin cancer cells in vitro. FCSPs also demonstrated immunomodulating effect by enhancing natural killer cells activity.

Conventional extraction technology for FCSPs involves treatment that is detrimental to its structural properties, yield and compositional attributes. Thus it hinders our understanding on the exact structure-function relations of FCSPs, as a consequence it limits commercial application possibilities for this valuable compound.

This PhD thesis delivers new understanding about the influences of extraction treatment parameters on the chemical nature of FCSPs and showed the most recent investigation of FCSPs as potential therapeutic agent for certain type of cancers.

The author of this PhD thesis is an agricultural engineer with MSc in agricultural development specialized in production and analysis of hydrocolloid from seaweed biomass for food and other commercial applications. He was involved in developing sustainable technology for algae bioremediation of wastewater and algae biomass production for bioenergy.

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